Does the cytoskeleton manipulate the auxin-induced changes in structure and motility of the endoplasmic reticulum?

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Abbreviations

BiP	Binding protein
CaMV	Cauliflower mosaic virus
CLSM	Confocal laser scanning microscopy
$DiIC_{18}$	1,1-di-octadecyl-3, 3,3', 3'-tetramethyl-lindocarbocyanine perchlorate
$DIOC_6(3)$	3,3`Dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribo nucleic acid
ER	Endoplasmic reticulum
F -actin	Filament actin
FLIP	Fluorescence loss in photobleaching
G -actin	Globular actin
GFP	Green Fluorescent Protein
HDEL	His-Asp-Glu-Leu
KDEL	Lys-Asp-Glu-Leu
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
pBIN	Plant binary vector
TEM	Transmission electron microscopy
UV	Ultra violet

Contents

Abbreviations…….……i

Contents…….……ii

List of Figures…….……....v

List of Tables……..…vi

Acknowledgements……...vii

Abstract……..…viii

Chapter 1 – Literature review of the cortical endoplasmic reticulum……..1

1.1 The endoplasmic reticulum…….....1

1.2 The Functions of ER…….....4

1.2.1 Functions of cortical ER…….……..4

1.2.2 Cortical ER as structural support…….….5

1.2.3 Cortical ER and signal transduction.…….……5

1.3 Ultrastructure of the cortical ER in plant cells…….…….....6

1.3.1 Chemical Fixation.……...…….……6

1.3.2 Freeze substitution.……...8

1.3.3 TEM tomography……9

1.4.1 Observation of cortical ER *in vivo*……..9

1.4.2 UV microscopy……..10

1.4.3 Carbocyanine dyes…….11

1.4.4 Green Fluorescent Protein as an alternative probe for ER 13

1.4.5 Confocal laser scanning microscopy…….15

1.5 GFP was used to establish ER continuity…….....16

1.6 GFP helped study changes in ER structure with response to growth.……....16

1.7 Cytoskeleton and ER…….....18

1.7.1 Microtubules and cortical ER…….19

1.7.2 Actin and ER……...…...21

1.8 Aim……...…….....23

Chapter 2 – Materials and Methods……...……....24

2.1 Plant culture……..…….…….....24

2.2 Plant dissection……...……..24

2.3 Observation using CLSM…….……27

2.4.1 Auxin treatment…….…….28

2.4.2 Latrunculin treatment……...……..28

2.4.3 Oryzalin treatment……..29

2.5 Quantifying the structure of the cortical ER…..……...……..29

2.6 Quantifying changes in ER structure over time…….30

2.7 Quantifying ER motility…….31

Chapter 3 – Results……...……...……35

3.1 Structure of the cortical ER network……..…….…….....35

3.2 The tubular ER network was dynamic……...……38

3.2.1 Dynamic equilibrium of the ER network..……...……..55

3.3 The structure and motility of cortical ER changed with cell growth…….....58

3.4 Auxin-induced growth of non-growing epidermal cells……67

3.5 Auxin treated cells underwent changes in ER structure and motility……..67

3.6 Disruption of the microtubules……..……70

3.7 Disrupting actin microfilaments caused tubules to be lost……71

Chapter 4 – Discussion..……...……...……75

4.1 The structure of the cortical ER was best described as a Dynamic equilibrium……..…….....75

4.2 The growing cells had different ER structure and dynamics……76

4.3 Perturbation of the cytoskeleton changed the cortical ER lattice…….78

4.4 The nuclear envelope may be an ER organizing center……82

4.5 Conclusion…….83

References..……...……...……84

Appendix 1 – Preparation of pea growth media……...…….A 1

Appendix 2 – Statistical analysis (ANOVA and Tukey Test) Comparison of the proportion of cortical ER between the growing and non-growing cells with the auxin treated non-growing cells ……..A 3

Appendix 3 – Statistical analysis (ANOVA and Tukey Test) for the area covered by the cortical ER in the treated and untreated cells……A 6

Appendix 4 – Statistical analysis (ANOVA and Tukey Test) of the average motile proportion of cortical ER in both treated and untreated cells over 60 seconds time frame……...…….A 9

Appendix 5 – Statistical analysis (ANOVA and Paired Sample t Test) for the dynamic equilibrium of the cortical ER……...…A12

Appendix 6 – Statistical analysis (Paired Sample t Test) for difference in the cell size of non-growing and auxin treated cells……...…...…A14

List of Figures

Figure 1 Typical epidermal plant cell……..3

Figure 2 The pea plant (*Pisum Sativum*)……26

Figure 3 Time series of binarized, confocal laser scanning Images of the non-growing cell showing the cortical ER……33

Figure 4 Confocal laser scanning microscopy image of the endoplasmic reticulum of the epidermal cell of 0.5µm thick section of pea plant tendril. The focal plane is in the cytoplasm at the cell periphery……..37

Figure 5 The ER motility shown by image overlay method……..40

Figure 6 Sliding of tubule junction……42

Figure 7 Lateral sliding and fusion of tubules occurred and retracted to reduce the number of polygons……...44

Figure 8 New tubules emerged, extended and coalesced with the existing tubular ER array, thus, splitting an existing polygon into two new polygons……49

Figure 9 New polygons were also formed by tubular branching in non-growing cells……...51

Figure 10 The severing of tubule and its subsequent absorption into the peripheral polygonal ER network of a growing epidermal cell……...54

Figure 11 The tubules attached to the nuclear envelope are dynamic……57

Figure 12 Comparison of the cortical ER and nuclear envelope of auxin treated non-growing and growing cells…….....60

Figure 13 Comparison of the proportion of cortical ER between the growing and non-growing cells with the auxin-treated non-growing cells……..……..….62

Figure 14 Comparison of the proportion of the cortical ER occupied in the growing, non-growing and treated non-growing cells……..64

Figure 15 Comparison of the average motile proportion of cortical ER in both treated and untreated cells over 60 seconds time frame……..66

Figure 16 The change in cortical ER structure in response to microtubule disruption using oryzalin…….69

Figure 17 The change in cortical ER structure in response to actin microfilament disruption using latrunculin B…….……73

List of Tables

Table 1 Measurement of tubule and lamella in treated and untreated epidermal cells ……45

Table 2 The tubular dynamics exhibited by the cortical ER tubules ……47

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Abstract

The variations in ER structure and motility under different stages of cell development remain largely unexplored. Here, I observe ER structure and the changes that take place in this structure over time in growing and non-growing live epidermal cells of the pea tendril. The ER was labelled by green fluorescent protein, fused to the KDEL-ER retention signal and confocal scanning laser microscopy was used to localize the fluorescent signal. I found both the structure and motility of growing cells to be different to non-growing cells. The growing cells had a more open arrangement of the cortical ER, fewer lamellae and showed greater tubular dynamics, while the non-growing cells had a denser arrangement of the cortical ER network, with more lamellae and less tubular dynamics.

Furthermore, these differences in the cortical ER structure and dynamics were due to growth as, the ER in non-growing cells showed characteristics similar to those seen in growing cells when these cells were induced to grow by the exogenous application of auxin. These changes in ER structure and dynamics were dependant on both the microtubules and actin cytoskeleton networks.

CHAPTER 1

Literature review of the cortical endoplasmic reticulum

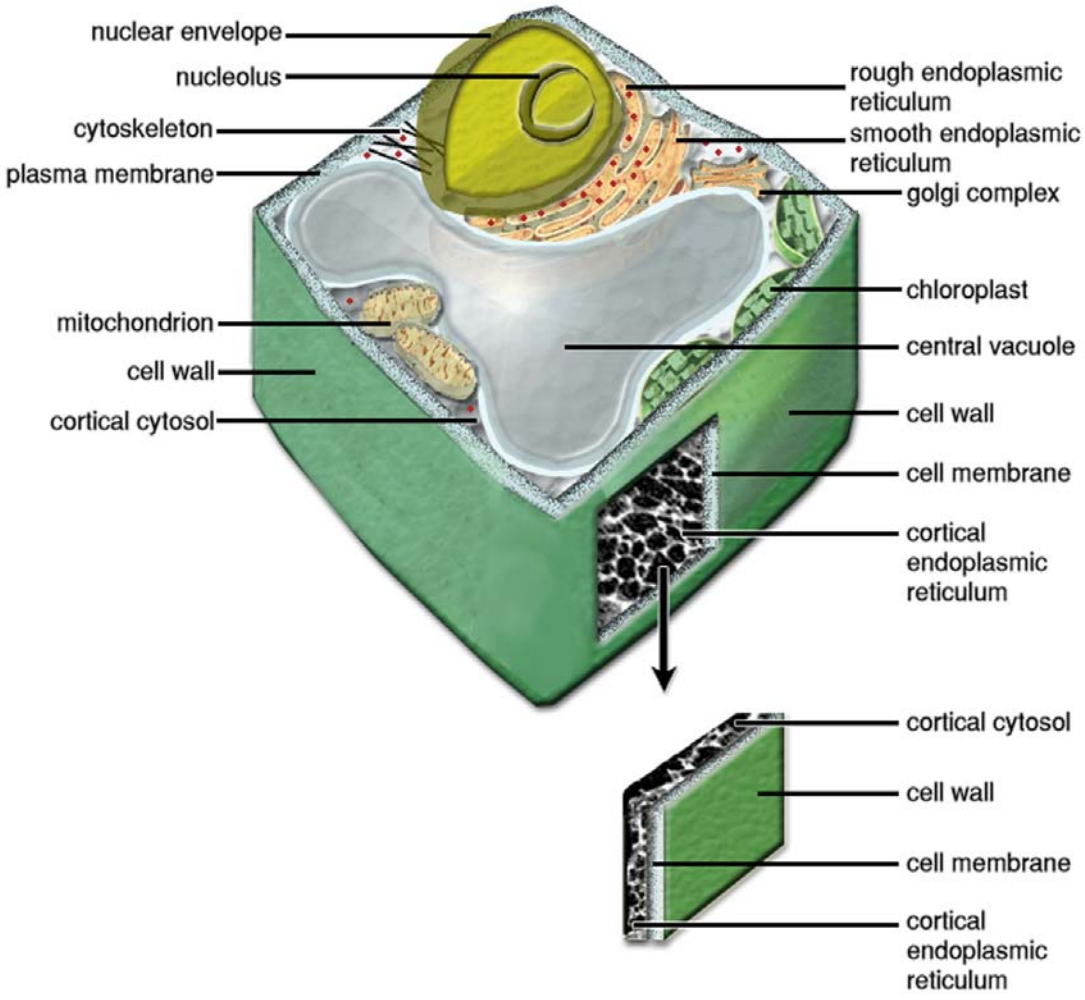
1.1 The endoplasmic reticulum

The endoplasmic reticulum (ER) is the largest intracellular organelle in terms of surface area and accounts for more than half the total membrane in many eukaryotic cells. It is characterized by its striking morphology of an inter-connected, three-dimensional network of tubules and lamellar sacs that underlie the plasma membrane, and connect with the nuclear envelope (Fig 1) (Porter *et al.*, 1945; Palade, G.E, 1974 and Lee and Chen 1988). This means that there is a single continuous lumen between the ER and the nuclear envelope since the ER is continuous with the outer nuclear membrane. Hence, the nuclear envelope is functionally a part of the ER network (Porter 1960, b). The degree of development of the reticulum and the relative proportions of its tubular and lamellar elements vary greatly between different cell types and within the same cell type in different phases of the physiological activity.

ER, by virtue of forming an endomembrane system, participates in the exchange of membrane molecules by diffusion or vesicle transportation. The functions that the ER performs are in congruence with the variation in its structure and location within the cell. Despite being continuous in nature, based on the presence of ribosomes, the ER is divided into two structurally different types, rough ER and smooth ER. In most plant cells, the ER is localized in the periphery of the cell in the cortical cytoplasm. For this reason, it is referred to as the cortical ER.

Figure 1. Typical plant epidermal cell

An epidermal plant cell is shown along with its various organelles in the cell cytoplasm enclosed by the plasma membrane and the cell wall. The cortical ER is the three dimensional network of tubules in the cytoplasm underlying the plasma membrane as shown by the cross section extending from the cytoplasm to the cell wall.


nuclear envelope
nucleolus
cytoskeleton
plasma membrane
mitochondrion
cell wall
cortical cytosol
rough endoplasmic reticulum
smooth endoplasmic reticulum
golgi complex
chloroplast
central vacuole
cell wall
cell membrane
cortical endoplasmic reticulum
cortical cytosol
cell wall
cell membrane
cortical endoplasmic reticulum

This is in contrast to a typical animal cell where ER extends throughout the cytosol. Since this thesis investigates the cortical ER in the plant epidermal cells, similar studies based on plant cells are discussed. References to the cortical ER in other eukaryotic cells are made when appropriate

1.2 The functions of ER

The ER, like most membranes, acts as a barrier, against entry and exit of molecules that cannot permeate the lipid bilayer. It also performs a range of different functions that include the synthesizing, processing and sorting of proteins, synthesizing a diverse array of lipids molecules, the storage of calcium ions in the ER lumen and regulation of their release into the cytosol. Additionally, it provides an anchoring site for actin filament bundles that drive cytoplasmic streaming in plant cells (Palade, G.E., 1974; Matlack *et al*., 1998; Meldolesim and Pozzan, 1998; Ma and Hendershot, 2001; McMaster, 2001 and Rapoport *et al*., 2002).

A majority of the functions performed by the ER are similar in most eukaryotic cells.

1.2.1 Functions of cortical ER

Since the cortical ER in plants is the largest part of the ER network, it is thought to perform most of the plethora of prominent ER functions including protein synthesis, lipid synthesis and their respective transport (Chrispeels, 1980). In addition, due to its distinct structure and location it may be better suited to perform functions that are in concert with its uniqueness. The functions of the cortical ER have been elucidated based on its structure, cytochemical analysis and comparison with similar systems (Hepler *et al*, 1990).

1.2.2 Cortical ER as structural support

The role of cortical ER as structural support has not been well documented in plant cells, though there has been some indirect reports on the same. The physical proximity of ER and the cytoskeleton hints towards the cortical ER acting as a site for its attachment (Lichtscheidl *et al.*, 1990) and this is reaffirmed by the fact that cortical ER is more rigid than the ER found deeper in the cell. In addition, it may also provide an anchorage site to regulate the spatial distribution of organelles (Hepler *et al.*, 1990). Such an association has been reported in the chloroplast of characean algae (Palevitz and Hepler, 1975). The close association of the cortical ER may also help the cytoskeleton in the process of membrane fusion by trapping and fusing golgi vesicles in its network (Hepler, 1982).

1.2.3 Cortical ER and signal transduction

Due to the physical proximity of the cortical ER to the plasma membrane, it is presumed to perform the role of signal transduction by transferring signals from the cell wall to the specific sites within the cell (Robards, 1976; Gunning, 1982; Hepler, 1982 and Overall *et al.*, 1982). Since the ER maintains continuity with the respective adjacent cells, these pathways for signal exchange could well extend beyond one cell. These exchanges could range from simple phospholipid molecules to complex signals (Hepler *et al.*, 1990). For example, the release of calcium ions from the ER lumen can be regulated by the signals received from the plasma membrane (Berridge and Irvine, 1989 and Putney *et al*., 1989).

There have been studies on plants that support the idea that cortical ER participates in calcium regulation and calcium based signal transduction. Not only is the location of

calcium in the ER lumen confirmed cytologically (Wick and Hepler, 1980), but biochemical studies on isolated ribosomes also demonstrate the calcium release in response to the signalling agent inositol triphosphate ($InsP_3$) (Drobak and Fergusan, 1985; Perdue *et al.*, 1988).

1.3. Ultra structure of the cortical ER in plant cells

1.3.1 Chemical fixation

The structure of the ER as interconnected membrane, as we know of today, has been resolved largely with the use of TEM (Transmission electron microscopy). Observations of the cells using TEM have shown that the ER is present in most plant cells and its total amount in the cell varies along with its structure and distribution (Gunning, 1982). A typical cortical ER network as observed under a TEM in plant epidermal cells consisted of closely connected lamellae that expand in some places, forming interconnected tubules with ribosomes studded on its cytosolic surface. This network connects to the nuclear envelope while sharing its lumen with the perinuclear space. The ER is observed lying close to other organelles such as the mitochondria without being in direct contact with it (Fig 1). Although most of the morphological attributes of the cortical ER had been resolved using various techniques of cell fixation and their subsequent observation under TEM, artifacts may have been generated due to the need to fix cells chemically as part of the preparation

Using TEM, the earliest observations of the cortical ER showed it as discontinuous tubular strands. These plant samples under study were prepared with the help of fixative permanganate. This fixative disrupted the ER and failed to show its continuous nature (Porter and Machado, 1960 a, b). For instance, ER was often

described to occur in discrete groupings instead of one interconnected network. This could have been caused by the disruption of ER by chemical fixatives (Hepler *et al*., 1990). The cortical ER showed fewer discontinuities upon the use of other commonly used fixatives like glutaraldehyde and osmium tetroxide. These fixatives suffered from the poor ability to stain the fine tubular parts of the cortical ER, making it difficult to detect its extensive spread in the cytosol (Ledbetter and Porter, 1963). To enhance the visibility of the ER, post fixatives such as osmium ferricyanide (OsFeCN) (Hepler, 1981; Schnepf *et al*., 1982) and zinc iodide-osmium (Dauwalder and Whaley, 1973; Harris, 1979) were used. These contrasting agents made even the fine tubules of the ER visible when observed with TEM. These post fixatives, when deposited in the ER lumen, add contrast to the ER site under observation, as they are impregnable to electrons beamed onto them during TEM. This helped to study ER in detail with relatively fewer disruptions. TEM has been used to study ER in different plant parts, including stamen hair, guard cells, non-stomatal epidermal cells, root cells and moss caulonemata by (Hepler, 1981; Schnepf *et al*., 1982; Palevitz and Hodge, 1984; Conrad *et al*., 1986; Lancelle and Hepler, 1986) respectively.

Using, different enhancements in fixation methods, it was observed that the cortical ER is made up of a network of tubules and lamellae that are interconnected and located adjacent to the plasma membrane (Hepler, 1981). It was also found that not only was the cortical ER a single continuum in every cell, the ER of the adjoining cells was also interlinked via plasmodesmata (Robards, 1976). Although there is no conclusive, evidence that this interlink is through the lumen of the ER in plasmodesmata (Cantrill *et al.,* 1999). Further enhancement in the TEM technique

resolved the finer details of the cortical ER including the interconnected tubules along with their predominantly polygonal arrangement.

Hepler *et al*., 1990, brought an important aspect, the indicative bond between the cortical ER and the plasma membrane to light. This positioned the cortical ER to have properties distinct from the rest of the ER despite being interconnected. The use of this technique reported a few apparent disruptions in the ER continuity structure. This new technique involved attaching cell protoplast to a film and washing away of the cytoplasm using hypotonic medium for cell lyses. The negative staining of such films on the TEM grid revealed the cortical ER along with other organelles, as seen in guard cell protoplast of *Vicia faba* (Wiedenhoeft, 1985). The drawback of this method is that it can be used to observe the cortical ER only in the cell protoplast and that it could not prevent the cortical ER from the adverse effect of the cell lyses procedure.

1.3.2 Freeze substitution

Most chemical fixatives cause considerable damage to the ER, ranging from tubular swelling to total disruption and make the ER appear as disconnected irregular strands (Mersey and McCulley, 1978). Hence, other methods of fixing the cells for the observation of ER were explored. One such method showed the cortical ER to be lying much closer to the plasma membrane, as seen in the root cells of lettuce (Lancelle *et al*., 1986). This was observed using the freeze substitution method of cell fixation. In this method, the sample is cooled rapidly to freeze all the structures instantly to avoid alterations and consequently preserve them in a life-like state. Although it involved the use of osmium for chemical stabilization, this reaction occurred at -80^{o} C, thereby reducing the harmful effect of this fixative. However, this

method too had disadvantages that included long duration of time required for substituting, the production of ice crystals that damage the ER and the reduced contrast of the images obtained.

1.3.3 TEM tomography

The major drawback of interpreting the TEM image stems from the fact that each image is derived from a single ultra thin section of the cell. This makes it difficult to interpret the three dimensional nature of the ER from a series of two-dimensional images (Staehelin, 1997). This difficulty has been largely overcome with the advent of TEM tomography that demonstrates, as expected, the ER as one inter-connected array in cells with a continuous lumen that includes the perinuclear space and also highlights the three dimensional ultra structure of the ER in plant cells (Fernandez *et al*., 2002). Unfortunately, this technique is both time consuming and expensive hence, only a few studies reportedly used this technique.

Despite the different enhancements in the way the samples are prepared for TEM observation, the limitation that remained unaddressed was that all the methods essentially involved killing of the cell. Hence, the nature of the cortical ER in a live cell remained elusive.

1.4.1 Observation of cortical ER *in vivo*

To understand the dynamic nature of the ER it was necessary to study its structure in live cells.

1.4.2 UV microscopy

The cortical ER has fine tubules yet, their dynamics can be observed *in vivo* because, their refractive index is different compared to the surrounding cytoplasm (Hepler *et al*., 1990). Taking advantage of this fact, a study was conducted on the morphological changes in the ER of the onion epidermal cells *in vivo* using ultra violet microscopy (UV-microscopy) (Url, 1964). In this study, the dynamic nature of ER was analysed in detail. The different tubular movements, including the breaking and rejoining at different sites of the cortical ER, were reported. This was the first attempt at using UV-microscopy for the cytomorphological study of the cortical ER. In this method, the ultra violet light used by cell components, such as proteins and nucleic acid, are observed in sharp contrast relative to the cytoplasm due to the diffraction of light. In a similar study on epidermal cells of onion *in vivo,* ultraviolet microscopy was used (Lichtscheidl and Url, 1987). This study reported similar ER dynamics and indicated the motion of the ER tubules to be along the F-actin microfilaments of the cytoskeleton. Increase in the use of digital methods of imaging has further enhanced UV-microscopy. Using these methods, the cell wall generated visual noise is reduced to get a clearer image. This technique was used to identify the difference in the morphology of the cortical ER. For example, observational studies of the onion cell reported the polygonal arrays of the cortical ER that appeared to be continuous with the tubular lamellae and were deeper in the cytoplasm (Lichtscheidl and Weiss, 1988). The difference in apparent movement was also reported. The movement of the tubules in the ER was reported to be Brownian, while the whole ER network showed lateral movement at a slow rate (Allen and Brown, 1988; Lichtscheidl and Weiss, 1988).

This observed dynamics of the tubules and the whole ER network is debatable as there could be an artifact caused by the harmful UV radiation. There were fewer studies using such methods because of the obvious difficulty in capturing the images from the microscope. This could be resolved to some extent by the use of UV-sensitive cameras. However, there has been little improvement in controlling the damaging effect of the UV rays to the samples under study, forcing a reduction in the observation time (Lichtscheidl and Url, 1987).

1.4.3 Carbocyanine dyes

The $DIOC_6(3)$ dye, a short chain, lipophilic carbocyanine dye, contributed to a large extent to the observational study of ER in live cells, as it did in fixed cells (Terasaki *et al*., 1986, Terasaki and Jaffe, 1991., Terasaki 1990). It permeates the plasma membrane and accumulates in the intracellular membrane. Hence, many internal membranes are labelled and consequently, the location of the ER has to be inferred from its structure and location in the cell. The fluorescent nature of the $DIOC_6(3)$ dyes has helped reveal successfully the interconnected nature of the ER with its tubules and lamellae when used in conjunction with epifluorescence microscopy (Quader and Schnepf, 1986). This technique was based on the principle of using a fluorescent dye in the sample under observation so that it fluoresces on excitation. This technique did help in the observational studies of cortical ER *in vivo*. However, this method could prove useful only for brief observations, as prolonged exposure to the excitation beam appeared to inhibit ER movements in the cell (Lee and Chen, 1988).

During the study of onion epidermal cells using the $DIOC_6(3)$dye, two distinct morphologies of ER were identified, viz. the regular polygonal tubular ER network in the peripheral cortex and long tubular strand- like ER in the interior region of the cell that remain interconnected (Quader and Schnepf, 1986). Most of these studies have used $DIOC_6(3)$, while others have used chlortetracycline dye that is a probe for membranes associated with calcium transport. Using this technique, groups have shown that cortical ER network contains calcium stores in the lumen (Berridge and Irvine, 1989. The dynamics of the cortical ER tubules were observed and the movement was classified as sliding and branching, breaking and fusion of tubules with this labelling technique (Dabora and Sheetz, 1988; Lee and Chen, 1988; Sanger *et al*., 1989). Although, with the help of the $DIOC_6(3)$ dye, coupled with digitally enhanced images, a greater understanding of the tubular dynamics was attained, there were still some distortions in ER structure and motility due to continuous exposure to radiation (Lee and Chen 1988).

Other lipophilic dyes such as DiI ($DiIC_{18}$) (1,1-di-octadecyl-3,3,3',3'-tetramethyl-lindocarbocyanine perchlorate), are weakly fluorescent dyes in water but fluoresce better when incorporated into membranes. The high transmission of infrared light through cells and tissues and low level of autofluorescence in the infrared make DiI useful as an *in vivo* tracer. This dye has to be microinjected into cells, as it does not cross the plasma membrane. Hence, it seems to have better specificity for ER as it diffuses and accumulates in continuous membranes (Terasaki and Jaffe, 1991). The argument that DiI labels the ER is based on the reasoning that it only diffuses in continuous membrane bilayers. So any discontinuous parts of the ER could not be observed. In addition, it was noticed that over a period there was significant labelling

of organelles other than ER, probably due to dye transfer from ER to the Golgi apparatus (Terasaki and Jaffe, 1991).

While the use of these different fluorescent dyes provides a first hand glimpse of the dynamic nature of ER, there were a few shortcomings of this technique. Firstly, the dyes were not specific and labelled organs other than ER (Terasaki and Jaffe, 1991). Secondly, there was some evidence of toxicity, either due to the probe itself or photobleaching of the probe (Quader *et al*., 1987).

1.4.4 Green Fluorescent Protein as an alternative probe for ER

The difficulties inherent in the DiI and $DIOC_6(3)$ labelling techniques can be largely overcome by the use of Green Fluorescent Protein. GFP is a protein involved in jellyfish bioluminescence that has proven to be a very versatile marker in many organisms (Chalfie and Kain, 1998). GFP is a 27-kDa, stable protein of 238 amino acids (Prasher *et al*.,1992) that emits green fluorescence when excited with blue or UV light (Inouye and Tsuji 1994). Proteins are retained in the lumen of the ER depending on the presence of specific retention sequences. In many soluble resident proteins of the ER molecular tetrapeptide sequences HDEL and KDEL have been identified at the C-terminal ends as part of their primary amino acid sequence, these serve as the retention signals (Brandizzi, 2004). GFP has been targeted to the ER lumen by making a chimeric protein construct with the GFP attached to any one of the ER retention sequences (Munro and Pelham 1987).

Proteins synthesized within the ER are transported to the Golgi apparatus as part of initiation towards biosynthetic-secretory pathway. The Golgi apparatus functions as a

sorting site before further transport (Alberts *et al*., 1994). All correctly folded proteins are retained in the Golgi, while the resident ER proteins are returned to the ER. This function of returning the proteins to the ER is performed by specialized receptors that recognize and retrieve the KDEL or HDEL sequences tagged soluble ER resident proteins. These receptors also regulate the retrograde transport from Golgi apparatus to the ER. (Semenza *et al.,*1990; Farquhar and Palade 1998 and Martinez-Menarguez *et al.,* 1999). Hence, proteins containing the constructs GFP- KDEL or GFP-HDEL remain concentrated in the ER because, if transported to the Golgi apparatus they are returned to the ER via retrograde transport.

There was a doubt whether GFP would be able to fold correctly within the ER, as the lumen environment can be more oxidizing than the cytosol (Hwang *et al*., 1992). However, fluorescence patterns that developed showed that this was not the case. Large membrane sheets and tubules of the cortical ER, along with the nuclear envelope, were labelled. Reports show that GFP was successfully targeted to the ER and that it folds correctly (Haseloff *et al*., 1997). The GFP as a marker has a significant advantage over the DiI method as in this method the fluorescence appears to remain specific to the ER, besides having favourable properties such as low toxicity and high stability.

There are some possible limitations of the GFP as a marker for the ER. In some instances, parts other than ER have been labelled as reported in the case of the fusiform bodies of the *Brassicaceae* (Gunning, 1998 and Ridge *et al*., 1999). For a long time the opinion prevailed that, GFP was cytotoxic to plant cells. It is still inconclusive as to whether the GFP is toxic to plant cells, as there are still reports like

(Liu *et al.*, 1999) which showed an association between GFP and apoptosis in mammalian cells. Again it remains to be proved whether this was a feature specific to mammalian and/or animal cells, as past research indicates that plants are morphologically and physiologically more adapted to deal with photonic or free radical damage as compared to animal cells. Sometimes some parts of the ER might exclude GFP and be unlabeled as reported in animal cells (Terasaki *et al.*, 1996). However, observations made in plant cells did not report this difficulty (Haseloff *et al.*, 1997; Gunning, 1998; Ridge *et al.*, 1999 and Matsushima, 2003). Despite these limitations, GFP is widely accepted for observing the dynamics of ER organization in different cells (Terasaki *et al.*, 1996).

1.4.5 Confocal laser scanning microscopy

The GFP method of labelling the ER is gaining popularity specially when used in conjunction with confocal laser scanning microscopy (CLSM). Confocal microscopy has been widely used and preferred over epifluorescence microscopy as confocal microscopy generates optical sections of fluorescently labelled samples. This makes it ideal for cells labelled with GFP.

The problem of detecting GFP by conventional epifluorescence microscopy is auto fluorescence, especially during *in vivo* observations. For example, plant cell walls and chloroplasts have high auto fluorescence. This problem can be overcome by confocal microscopy as it generates optical sections of a fluorescently labelled sample coupled with the use of narrow band emission filters that are specifically selected for GFP fluorescence (Haseloff and Amos, 1995).

High-resolution optical techniques are used to observe the dynamic activities of the ER, using specialized microscope objectives and the optical sectioning properties of the confocal microscope (Haseloff and Amos, 1995). It is possible to monitor easily and with great precision, both the three-dimensional arrangement of the ER *in vivo* and the dynamics over a small gap of time with the help of time-lapse series of images.

1.5 GFP was used to establish ER continuity

Evidence for a single continuous ER network was provided by experiments using GFP and FLIP (fluorescence loss in photobleaching). GFP proteins were targeted to the lumen or the membrane of the ER expressed in a range of cells and imaged by fluorescence microscopy. By focussing the laser onto the subset of the GFP-labelled ER, it was possible to photobleach the GFP in the illuminated region. If the process was stopped mid way through, it was observed that parts of the ER not exposed to the laser were also diminished in GFP fluorescence. This indicated that GFP molecules from outside the photobleaching zone were continually moving into this region. To photobleach the ER in the selected region, it was necessary to photobleach the GFP from the entire ER network. This demonstrated that all GFP-tagged molecules were free to diffuse within an inter-connected membrane system towards the photobleaching zone (Cole *et al*., 1996; Subramanian and Meyer 1997; Dayel *et al*., 1999 and Terasaki, 2000).

1.6 GFP- helped study changes in ER structure with response to growth

Most observations of ER in live cells have concentrated on describing the distribution within the cell and the dynamic nature of the ER network (Url, 1964; Lichtscheidl and

Url, 1987; Dabora and Sheetz, 1988; Lee and Chen, 1988 and Sanger *et al*., 1989). The ER was dynamic and movements such as tubular ER branching, breaking and fusion were seen. These observations supported those made earlier using $DIOC_6(3)$ and DiI.

With GFP, it has been possible to observe the changes in the ER that occur in different physiological states of a single cell type. Ridge and co-workers (1999) described the difference in the ER structure between growing and non-growing cells in growing root tip of *Arabidopsis thaliana*. In this study it was observed that the density of the tubular arrangement and the number of lamellae in the ER network were different in growing and non-growing cells. The cortical ER, as seen in the growing cells (root tip end), formed extended sheets with perforations, characterized by increased fluorescence. This pattern of cortical ER changed abruptly to large perforations and the sheet-like membranes reduced to tubules in the non-growing cell-zone (basipetal end). It remains to be confirmed whether this difference in the cortical ER structure is special to the root cell and/or is generic to the *Arabidopsis spp*. The root hair showed a similar arrangement of cortical ER in its entire length as in growing root tip throughout its growth period and its ER pattern reversed to that observed in non-growing cell on cessation of growth (Ridge *et al*., 1990). This gave further evidence that the structure of the cortical ER was growth-directed. It is necessary to conduct assays in which growth is induced to confirm whether the change in the morphology and dynamics of the cortical ER observed in the cells was directed by growth. This study also demonstrated the dynamic nature of the cortical ER but failed to classify the tubular movement like those described in the studies of the onion cells (Quader and Schnepf, 1986; Lichtscheidl and Url, 1987 and Knebel *et*

al., 1990). In addition, it did not demonstrate the difference, if any, in the cortical ER dynamics and the morphology of the nuclear envelope between the growing and the non- growing cells.

There are reports of a link between the ER and the auxin content in the cortical cells of *Kalanchoe daigremontiana* indicating more ER in auxin producing cells (J.Lipetz, 1967-1970,). An earlier study reported that the cells grown in the presence of auxin change their ER architecture completely as opposed to cells grown without auxin (Stickens, 1996). Since growing plant cells have a higher level of auxin, this may well support the idea that ER network is different in growing and non-growing cells.

1.7 Cytoskeleton and ER

The advent of the electron microscope not only helped in identifying the finer structures like the ER in the cytoplasm but also helped in observing a very important aspect of the cell, its cytoskeleton. The cytoskeleton was observed in the cytoplasm and was found to perform the important function of maintaining cell structure, generating cell motility and supporting the organelles within the cell, these functions were performed by the various components of the cytoskeleton as described (Alberts *et al.,* 1994). In most eukaryotic cells, the most commonly observed cytoskeletal filaments are microtubules that determine the spatial distribution of membranous organelles and control their intracellular transport, actin filaments that maintain the overall shape of the cell and are responsible for their overall locomotion and intermediate filaments that provide the cell with mechanical strength (Alberts *et al.*, 1994). Given that, the ER is an extensive and motile organelle, it seems likely that the cytoskeleton could probably be involved in regulating its structure and motility. In

various cytoskeleton and cortical ER related studies it was found that, there was not only a close association between the ER and the plasma membrane but also between the actin filaments (Lee *et al*., 1989)

1.7.1 Microtubules and cortical ER

Microtubules are a dynamic part of the cytoskeleton that participates in many important cellular processes (Desai and Mitchison, 1997). In plant cells, the microtubules form a cortical array lying just inside the plasma membrane (Ledbeter and Porter, 1963). These cortical arrays of microtubules are reported to be involved in directing the deposition of cellulose that plays an important role in cell growth (Lloyd, 1991; Giddings and Staehlin, 1991 and Cyr, 1994). The spatial organisation of microtubules in the plant cell cortex is a process that involves the interaction between different complexes and auxiliary molecules. It is controlled by the combination of various factors e.g. chemical factors such as plant hormones and physical factors like light (Iwata and Hagetsu, 1989; Shibaoka, 1994 and Marc *et al.,* 1998). These factors can modify the orientation of the microtubules during cell growth. The microtubules may be linked to each other and to different organelles with the help of microtubule-associated proteins (Cyr, 1991; Hirokawa, 1994 and Mandelkow and Mandelkow, 1995). These proteins are also known to have an impact on the microtubule stability and consequently, affect their organization and dynamics (Hirokawa, 1994 and Desai and Mitchison, 1997).

Studies have shown the formation of the new ER tubules and their movement along the tracks of already formed microtubules (Lee, Fergusan and Chen, 1989), hinting towards their possible role in ER tubule formation. However, since observations in

this study were made using dyes that can cause aberrations on continuous exposure to the UV radiations used in the microscopy, this could cause anomalies and show distortions not normally observed in a live cell. Despite several attempts to establish a link between the microtubules and the ER, there has been no conclusive evidence of the role of microtubules in controlling the structure and/or motility of the cortical ER in plant cells. One of the reasons is that the microtubules, despite being in close proximity to the ER, are not co-aligned to them. For example, the epidermal cells containing reticulate ER network may have microtubules arranged helicoidally near them (Quader *et al*., 1987). In other studies on plant cells, it was found that cortical microtubules were arranged parallel to one another and perpendicular to the longitudinal axis of the cell that was different from the lattice-like arrangement of the cortical ER network (Quader and Schnepf, 1989; Quader *et al.*, 1987; Lichtscheidl and Url, 1990 and Knebel *et al.*, 1990).

In addition, contrasting results of the change in structure of the ER network following microtubule disruption are reported. One study claims that the ER network is disrupted by colchicines (a microtubule depolymerising agent) (Allen and Brown, 1988), while in another study using oryzalin and trifluralin drugs that are more specific to plant microtubule disruption, no impact on the cortical ER network was reported (Quader *et al*., 1989). Besides, few groups argued about the involvement of microtubules supported by actin in the ER network distribution in plant cells (Samaj *et al.*, 2000 and Terasaki *et al*., 1986).

There has been no conclusive evidence of the role of microtubules in the dynamics of the cortical ER in plant cells. The *in vivo* studies in animal cells hint the involvement

of microtubules due to their co-localization with the cortical ER in the animal cells (Dabora and Sheetz 1988; Allan and Lane, 1999; Dreier and Rapoport, 2000 and Voeltz *et al*., 2002).

1.7.2 Actin and ER

Actin is an integral part of the cytoskeleton along with the microtubules. It occurs in plant cells either in the form of monomers (G-actin) or a combination of many monomers, to form actin filaments (F-actin). The F-actin is distributed in the cortical cytoplasm of the growing plant cells (McCurdy *et al.*, 1988; Hasezawa *et al.*, 1989; Jung and Wernicke, 1991 and Baluska *et al*., 1997). It also plays an important part in cytoplasmic streaming (Palevitz *et al*., 1974). This streaming is reported to be directed by the polarity of the F- actin filaments (Sheetz and Spudich, 1983). The F-actin is bound by actin binding proteins, that provide binding sites for several other molecules besides actin (Ren *et al.*, 1997) e.g., molecular motor protein myosin (Asada and Collings, 1997). Several studies involving the use of cytoskeletal drugs show that actinmyosin complexes are involved in endocellular movement of ER in higher plant cells ((Kachar and Reese, 1988; Quader *et al*., 1987, 1989; Knebel *et al*.,1990; Quader, 1990 and Liebe and Menzel, 1995). In plant cells, the relationship of the cortical ER to the actin cytoskeleton is based on the TEM studies that show the co-localisation of the ER with actin microfilaments (Quader *et al*., 1987; Lichtscheidl *et al*., 1990 and Lancelle and Hepler, 1989).

The cortical ER tubules are reported to share a common kind of linear movement as observed in the actin filaments that is similar to tracing the path of a point on a line. The *in vivo* studies indicate that the network formation of the actin is very similar to

that observed in the cortical ER network. Both formed a lattice-like array in the cell cortex. In plant cells, the notable example is observed in the *Characean* algae (Nagai and Hayama, 1979; Williamson, 1979 and Kachar and Reese, 1988), and in higher plants (Goosen-De Roo *et al*., 1983; Quader *et al*., 1987 and Lichtscheidl *et al*., 1990). The potential for the movement of the ER directed by actin was reported by a more advanced technique of high-pressure freezing-fixation followed by freeze-substitution (Lichtscheidl *et al*., 1990). This idea is supported by the *in vivo* studies that indicate the correlation between the ER network distribution and the actin filaments distribution not only in the cell cortex but also in the interior cytoplasm (Quader and Schnepf, 1989; Quader *et al*., 1987-1989; Quader and Fast, 1990; Lichtscheidl and Url, 1990 and Knebel *et al*., 1990).

Although most of these studies demonstrated the similarity in the actin filaments and ER tubules distribution, they failed to show the dependence of the ER distribution and movement on actin. In addition, contrasting results of the drugs have been reported. For example, there was a loss of ER tubular motility but no change in tubular formation after treatment with anti-actin drug, cytochalasin D (Knebel *et al*., 1990). While in another study, it was shown that the ER tubular formation ceased after recovery from cold shock treatment in the presence of cytochalasin D drug (Quader *et al*., 1989). It could well suggest that actin filaments may be required for tubular formation but may not play a role in the maintenance of tubular structure. This idea would oppose the earlier studies that hypothesized the actin filaments to support the ER network. Thus, the exact role of the actin filaments in directing the structure and/or motility remains to be confirmed.

1.8 Aim

In this thesis, I will investigate the relationship of the structure and dynamics of the cortical ER to the growth status of epidermal cells. I will be using a plant with GFP-labelled ER and will observe the ER in living cells with confocal laser scanning microscopy. This will make it possible to study the three dimensional distribution of the ER as well as follow changes in the ER structure that take place over time. I chose to study the epidermal cells in *Pisum sativum* (pea plant) as the epidermal cells of the outer wall of pea stem have proved to be a good medium for observing cell growth and the response of auxin treatments (Syndonia, 1993). The difference in the cortical ER structure and motility in growing versus non-growing plant cells will be compared. To determine if differences seen are due to growth, I will induce non-growing cells to resume growth with the exogenous application of growth hormone, auxin. ER in cells with auxin-induced growth will be compared to those that were naturally growing. The role of cytoskeleton in regulating the changes in ER structure will be investigated using cytoskeletal inhibitors.

CHAPTER 2

Materials and Methods

2.1 Plant culture

Pea plants (*Pisum sativum,* var. Crown) obtained from Dr. Jan Grant (Crop and Food Research Institute, New Zealand) was stably transformed with DNA encoding ER targeted green fluorescent protein (GFP) (Chapter 1, Section 1.4.4). The pBIN 35S-*mgfp*5-ER plasmid used to transform the plant contained the ER retention KDEL fused to the sequence for GFP. The cauliflower mosaic virus (CaMV) promoter and the gene for kanamycin resistance were also included in this plasmid. Dr. Haseloff provided the plasmid to Dr. Jan Grant and the description of the plasmid is given in Haseloff *et al.*, (1997) or at http://brindabella.mrc-1mb.cam.ac.uk

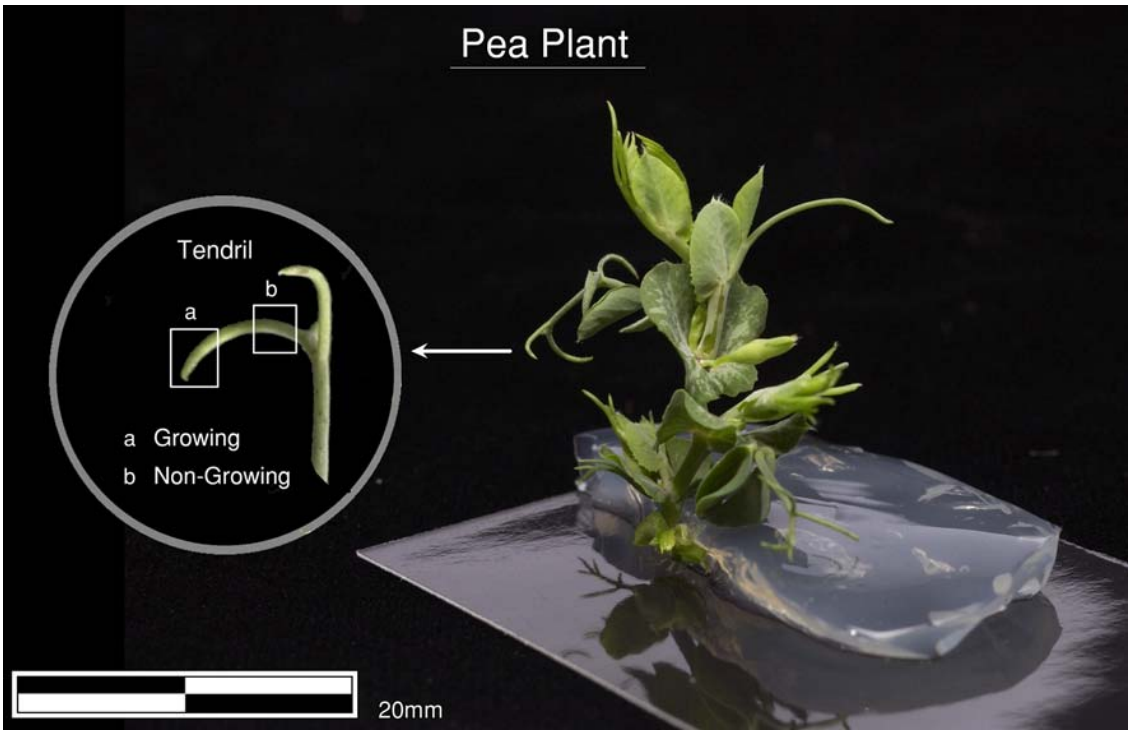
The shoots of the transgenic pea were grown in MS medium(Murashige and Skoog, 1962, Appendix 1) in a growth room at 20-24 degrees centigrade with 16 hours light and 8 hours of darkness. New shoot tissues were clipped from the plant and transferred to fresh MS medium every 3 weeks (Grant *et al*., 1995).

2.2 Plant dissection

I selected immature tendrils that were 2-5 days old, occurring in groups of three and lacking an apical hook for my experiments (Fig 2). I avoided older tendrils with apical hooks as by this stage of development the tendril showed thigmotropism (Riehl and Jaffe, 1984). I did not want changes in growth or response to touch and confound my experiment.

Figure 2. The pea plant (*Pisum sativum*)

Shown here is a 30 days old pea plant that has been stably transformed with DNA encoding GFP-KDEL sequence that ensures the retention of the GFP in the ER. The tendrils from this plant were used for the observational study of the cortical ER in the epidermal cells. To observe the ER in growing cells, tendrils were excised near the tip (a), and for the observational studies involving non-growing cells the tendrils were excised near the base (b).


Pea Plant
Tendril
a
b
a Growing
b Non-Growing
20mm

The 4-5 days old tendrils typically emerged in groups of 2 or 3 (Fig 2). I also did not use tendrils that occurred singly as the cell growth gradient was poor.

To study growing cells, I used 2-3 days old tendrils that were 1-2mm in length. Growing cells were sampled near the tip of the tendril. At this stage, all cells in the tendril tip were growing and consequently, being enlarged. Non-growing cells were observed in larger 5-6mm, 4-5 days old tendrils. These tendrils contained growing cells at the tip (Fig. 2 a) and the cells that had stopped growing near the base (Fig 2 b).

I also observed ER in the epidermal cells of the leaves and the stem. However, preparation of the cells required making epidermal peels. This often damaged the delicate cells destroying the ER structure. It is for this reason that I chose to work on the epidermal cells of the tendril. Tendrils were excised from the plants and placed in distilled water for 30 minutes prior to mounting on a slide and covering with a cover slip.

2.3 Observations using CLSM

The samples were examined using a BioRad MRC-600 laser-scanning confocal microscope linked to an Olympus IX-70 inverted microscope and 40 X oil immersion objectives (NA=1.4). The GFP proteins in the ER network were excited using the blue line of the argon laser and the fluorescent signals were filtered with a 500-530 nm band pass emission filter. Gain, offset and iris settings were held constant so that fluorescent intensities could be compared. Images were captured using the BioRad software. For each sample, a stack of optical sections (Z series) was collected at

0.5µm intervals. This was the method for observation for all the tendrils including those treated with auxin and cytoskeleton disrupting drugs. Time series of one focal plane of the cortical ER were also collected. For time series, cells were scanned every 10 seconds for 100 seconds.

2.4.1 Auxin treatment

Auxin (1-naphthaleneacetic acid or NAA, Sigma Chemical Company) was used to induce non-growing cells to resume growth. In order to determine the concentration of NAA required and the length of time needed to observe the change in ER structure, tendrils having non-growing cells were subjected to different concentrations of auxin (5µM, 10µM, 20µM, 30µM, 40µM and 50µM) and were observed after 30 minutes. It was found that 10µM was the lowest concentration that consistently induced a change in the ER structure in 30 minutes. To check whether this concentration also induced growth in the tendrils, a sample size of n=10 tendrils was immersed in 10µM auxin solution for 24 hours and observed. There was an increase in the cell size indicating that the cells had resumed growth.

2.4.2 Latrunculin treatment

To investigate the role of actin microfilaments in the growth induced change in the ER structure; cells were treated with the actin de-polymerising drug latrunculin B (Biomol Research Labs.,Inc). To determine the concentration of latrunculin B, 4-5 day old tendrils were excised from the plant and placed in petridishes containing different concentrations (0.1µM, 0.25µM, 0.5µM and 1.0µM latrunculin B in 0.1% DMSO dimethyl sulphoxide) distilled in water. It was observed that the 0.1µM of

latrunculin B was the concentration at which the ER structure showed a change without showing damaging effects. To show that the toxicity had not occurred, subsets of tendrils were washed in distilled water for 24 hours following the drug treatment. For observation of the effects of both auxin and latrunculin B, the mature tendrils were placed in 0.1µM of latrunculin B for 1 hour and then these tendrils were transferred to petridishes containing 10µM of Auxin for 30 minutes after which they were mounted on slides for observational study with CLSM.

2.4.3 Oryzalin treatment

The role of the microtubules in the growth-induced changes of the ER was investigated using oryzalin (Dow Agro sciences), a microtubule depolymerising drug. In order to find out the lowest concentration of oryzalin-required disruption of the microtubules, a regime similar to that used in latrunculin B treatment was followed. About 4-5 day old tendrils were excised and placed in petridishes containing different concentrations of oryzalin (5µM, 10µM, 15µM, 20µM, 40µM, 50µM and 60µM in 0.1% DMSO), distilled in water. To observe the effects of perturbation of microtubules along with auxin induced growth, the excised tendrils were placed in 10 µM of oryzlin for 1 hour. These tendrils were then transferred to petridishes containing10µM auxin for 30 minutes, after which they were mounted on a slide and observed using CLSM.

2.5 Quantifying the structure of the cortical ER

The proportion of the cortex occupied by ER was determined by dividing the area of the cortical ER by the total area of cortical cytoplasm (Image-Pro Plus version 4.5 for

Microsoft Windows, Media Cybernetics, Inc.). The inter-tubular area (the space in between tubules) was used to indicate the extent of the spread of the ER lattice. These regions were selected and their area measured using ImagePro.

2.6 Quantifying changes in ER structure over time

This analysis was based on visual observations made of the moving images collected from the time series obtained from CLSM. I made an observation on 500 μm^2 regions that was randomly selected in 10 cells for each of the treatments used respectively. The ER within this region was observed for the 100 seconds time course.

I made a note of the different type of tubular movements of ER in all cells, after that a visual observation of the frequency of occurrence of these tubular movements was recorded.

The movements scored were:

1. Branching- this described a new tubule emerging from an existing tubule
2. Extension of a branch- this described a newly emerging tubule becoming longer.
3. Retraction of a branch- this described a newly emerging tubule that was no longer extending but was shrinking while retracing its path of origin
4. Severing of tubule - this described the breaking of the tubule from its point of attachment and gradually shrinking in size until it is reabsorbed into the ER network.
5. Sliding of tubular junction- this described the point of attachment of tubules to slide along some unknown track in linear motion, this movement made the attached tubules to be pulled along the direction of the junction.

6. Lateral fusion of tubules- this described two parallel tubules sliding towards each other until they coalesce into one tubule, thereby reducing two polygons into one.
7. Lateral splitting of tubules-this describes a tubule splitting into tubules, laterally and forming two polygons.

2.7 Quantifying ER motility

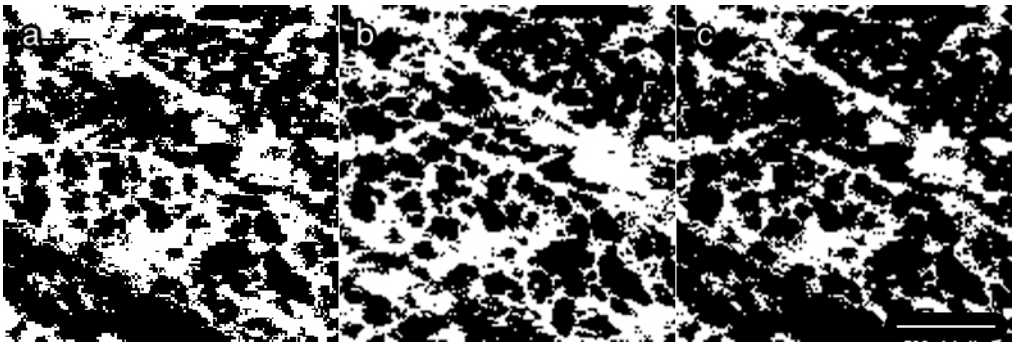
For motility measurements, the software Metamorph (Metamorph Imaging system version 2 for Microsoft Windows, Universal Imaging Corporation) was used along with the Image Professional software program. The motile part of the cortical ER was obtained as explained. Two sets of images were selected for different cells from a time series of confocal microscopy that were 60 seconds apart. The images were imported into the Metamorph software that was used to first binarize all the sets of images taken from each of the cell treatment. The first image from each set was assumed as (stationary) (Fig 3 a) and the second image taken after 60 seconds in the series was taken as the (motility image) (Fig 3 b). The motility image was then subtracted from the stationary image and resultant image (Fig 3 c) was obtained using the Metamorph software. These resultant images showed the parts of the cortical ER that were not motile. These resultant images were used to compare the extent of motility in the cells under observation using the ImagePro software as explained (fig 3).

The area covered by the cortical ER in the resultant images was measured using the Image Pro that indicated the non-motile parts of the ER during the 60 seconds observation.

Figure 3. Time series of binarized, confocal laser scanning images of the non-growing cell showing the cortical ER.

In order to measure the amount of motile cortical ER over a period, two images from a time series of non-growing cells with a time gap of 60 seconds were selected. The images were binarized using the Metamorph software. The first image (a) was assumed as stationary and the image obtained after 60 seconds was taken as the motility image (b). The image (b) was subtracted from the image (a) to get the resultant image (c). This resultant image showed parts of cortical ER that were not motile. To find the parts of the cortical ER that moved the resultant image (c) was subtracted from the image (a), for this calculation, the area of the cortical ER was measured using the ImagePro software. Note that the nuclear envelope was not included in this measurement.

Bar = 10μm


a
b
c

The area of the ER obtained in the resultant images was subtracted from the area covered by the ER in the stationary images. The area of cortical ER thus obtained showed the motile parts of the cortical ER during the 60 seconds. This area was converted into proportion as the size of the cells varied. This calculated proportion of area of ER was recorded for the different cell treatments as well as growing and non-growing cells. The comparison is represented graphically in Chapter 3, Fig 15.

The statistical analysis for all the readings was performed with the Statistix version Software for Microsoft Windows. The corresponding readings were recorded and calculated with Microsoft Office Excel 2003.

CHAPTER 3

Results

3.1 Structure of the cortical ER network

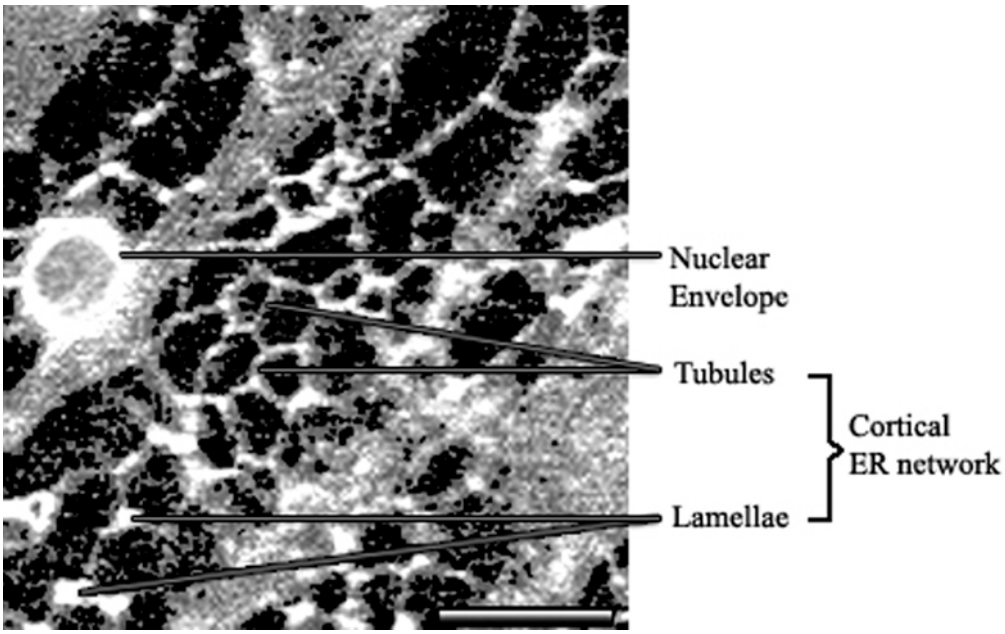
The structure, motility and distribution of ER in the epidermal cells of the pea plant tendril were observed *in vivo* using confocal laser scanning microscopy. The cortical ER network was a three dimensional labyrinth, lying adjacent to the plasma membrane in the cortical cytoplasm. It was continuous with the nuclear envelope as inferred by the presence of GFP fluorescence in the nuclear envelope and attachment of the ER tubules to the nuclear envelope.

The cortical ER was made up of a polygonal network of tubules, which interconnected with each other and with unevenly distributed flattened sacs of ER (lamellae) (Fig 4). This distribution was consistent in all cells (n=2380 cells) observed during the course of this project (Fig 4). There were three structurally distinct regions. Tubules of ER that ranged in width from (0.8 $\pm$ 0.2) µm to (2.7 $\pm$ 0.2) µm and in length from (5.0 $\pm$ 0.3) µm to (11 $\pm$ 0.10) µm, and flattened plate- like lamellar regions that ranged in diameter from (1.4 $\pm$ 0.2) microns to (5.0 $\pm$ 0.5) µm. The tubules and the lamella were interconnected and together formed the polygonal ER arrays of the cortical ER. The third region of the ER was the nuclear envelope (Fig.4).

The cortical ER was captured in a single confocal Z section of 0.5µm, thus it must have been thinner than 0.5µm. The only region where the network increased in thickness in the cytosol was adjacent to the nuclear envelope.

Figure 4. Confocal laser scanning microscopy image of the endoplasmic reticulum of the epidermal cell of 0.5μm thick section of pea plant tendril. The focal plane is in the cytoplasm at the cell periphery.

The predominant structure is the cortical endoplasmic reticulum network. It consists of a dynamic framework of inter-connected tubules of ER. These tubules join the ER lamellae that are plate-like structures that are less motile. The tubules are also linked with the nuclear envelope. In this micrograph, a median focal plane of the nucleus is observed. For this reason, the mid-section of the nucleus lacks fluorescence.
Bar = 10μ


Nuclear
Envelope
Tubules
Cortical
ER network
Lamellae

3.2 The tubular ER network was dynamic

ER tubules were observed to bend, move laterally, branch out to form new tubules as well as break and coalesce with the existing tubules. While the tubules showed the maximum motility, the lamellae and the nuclear envelope, rarely moved and when they did, they covered a small distance at a slow rate (Fig 5).

The most commonly observed movement was the sliding of tubule junctions (point of attachment of two tubules). This occurred when the tubule junction slipped along the attached tubules (Fig 6). This movement of the tubular junctions made the attached tubules slide in the same direction. Either one or both junctions of a given tubule moved (Fig 7 b-c). Such sliding of tubules often included a change in the tubule length (i.e. the tubule gets shorter in Figure 7 c-d). Note that the tubular diameter did not increase as the tubules shortened. Similarly, tubules did not narrow down when they increased in length with sliding (Fig 6).

The formation of new tubules by the branching of existing tubules occurred on an average (1+ 0.6 times in a 250µm2 area in a 100 seconds interval) (Table 2). Prior to the emergence of a new tubule, the branching tubule developed a bump or pre-branch (Fig 8 b-c and Fig 9 c-d) at the point that the new tubule would emerge. The new tubule emerged, and then extended across the polygon in a linear fashion (Fig. 8 d). The straight nature of the growing tubule indicated that it was either under tension from a pulling force or moving along the tracks of a lateral support structure (Fig 8 d and Fig 9 e). Eventually, the new tubule extended across the polygon and fused to the tubule on the opposite side, thus splitting the original polygon into two (Fig8f).

Figure 5. The ER motility shown by image overlay method.

The three images of the same region and focal plane of the epidermal cell were acquired at 30 seconds interval. The different images were given false colors (green b and red c). These two pseudo coloured images were laid one over the other to obtain the image d. This image d, showed parts of the ER that move and appear as one of the pseudo-colors or in the combination of the two colors as brown. The first section (a) that indicates no motility was then overlaid on the image d to show the parts of the ER that did not move. The nuclear envelope is shown as N and the lamellar region as L. Bar = 10μ

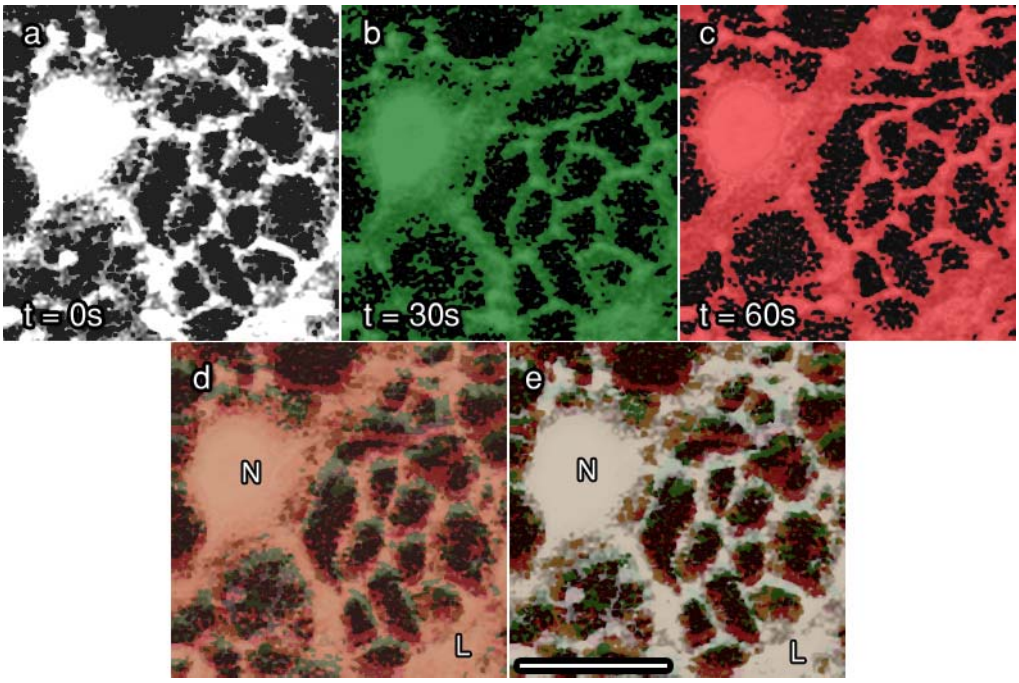

a
t = 0s
b
t = 30s
c
t = 60s
d
N
L
e
N
L

Figure 6. Sliding of tubule junction

The point of attachment of two tubules (tubular junction) appears to slide as indicated by the arrow in (a). This movement is predominantly observed in the cortical ER network. Sliding movement of the junction is responsible for the sliding of the tubules attached to it (b, c). The junction seems to slide along a track as it traces a linear path. In addition, it is noted that there is no apparent change in the thickness of the tubules as they are pulled due to the sliding action of the junction.

Bar = 5μ

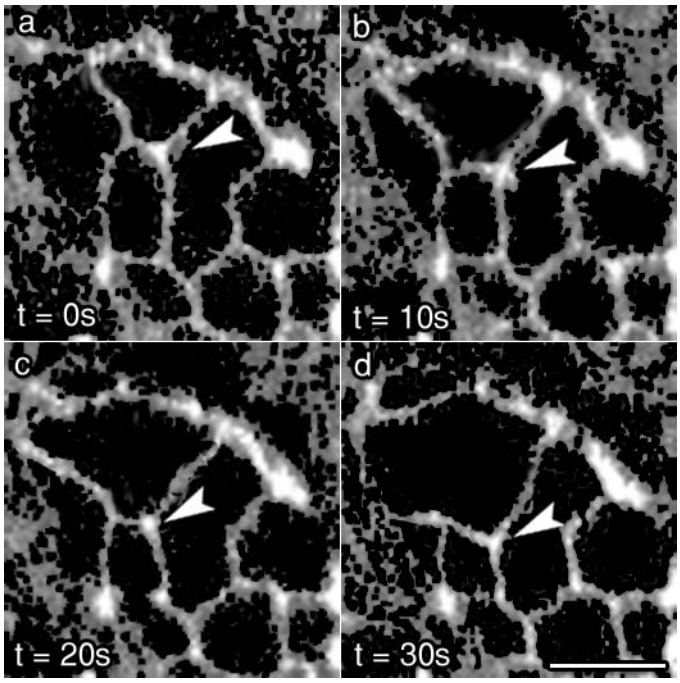

a
t = 0s
b
t = 10s
c
t = 20s
d
t = 30s

Figure 7. Lateral sliding and fusion of tubules occurred and acted to reduce the number of polygons.

The tubule indicated by the arrow (a-e) became mobile at the points of attachment to the tubule. Because of this, it slid along the tubule since its length became shorter and the fluorescence intensity remained similar. As it moved, it might have been reabsorbed into the attached tubules. Reabsorption appeared completed by the lateral fusion of tubules (f).

Bar = 5μ

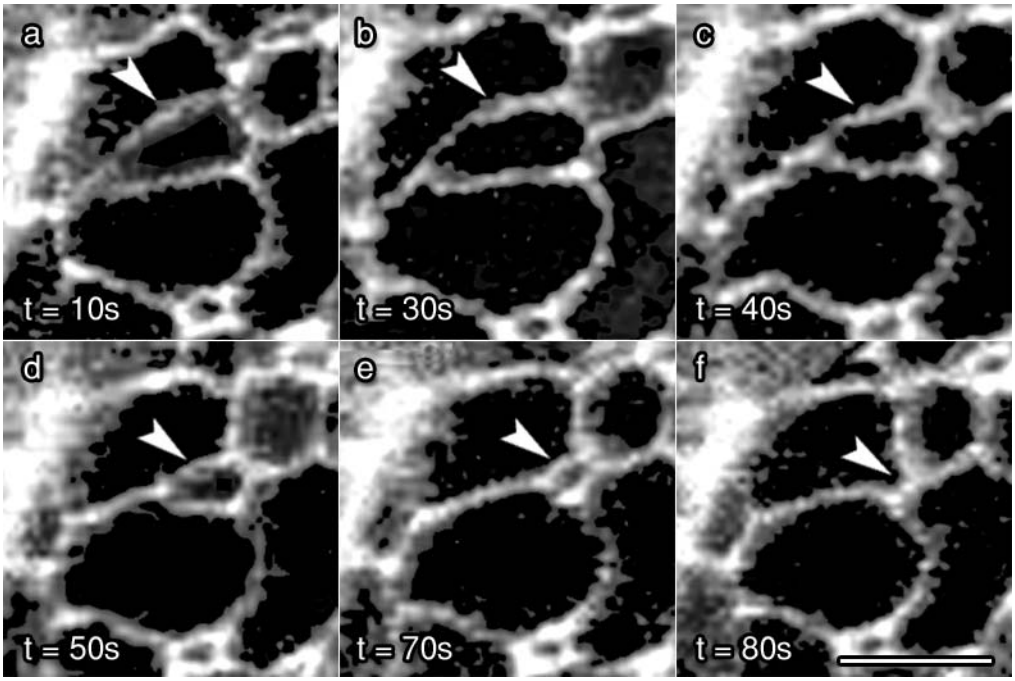

a
t = 10s
b
t = 30s
c
t = 40s
d
t = 50s
e
t = 70s
f
t = 80s

Table 1 Measurement of tubule and lamella in treated and untreated epidermal cells.

The tubular length and width along with the number of lamella and their diameter of the cortical ER was calculated using the ImagePro software on the images obtained from confocal microscopy. Cell treatments include, subjecting non-growing cells to various treatments that include, 10μM auxin (Non-growing + auxin), 0.1 μM latrunculin B and 10μM auxin (Non-growing + Latrunculin B+ auxin) and 20 μM Oryzalin + 10μM auxin (Non-growing + Oryzalin). The sample size of n =30 was taken for tubule measurement and lamellar width while an area of 250μm^2 was observed in each of the groups under study for the lamellae count.

Cell type/ treatment	Tubule length (Microns)	Tubule width (Microns)	Lamellae (Number)	Lamellar diameter (Microns)
Non-growing	5.0 $\pm$ 0.3	2.7 $\pm$ 0.2	4.44 $\pm$ 0.4	5.0 $\pm$ 0.5
Growing	11.0 $\pm$ 1.0	0.8 $\pm$ 0.2	2.82 $\pm$ 0.3	1.4 $\pm$ 0.2
Non-growing +auxin	7.3 $\pm$ 0.8	1.3 $\pm$ 0.2	3.38 $\pm$ 0.3	2.0 $\pm$ 0.2
Non-growing +Latrunculin B +auxin	6.8 $\pm$ 1.2	1.6 $\pm$ 0.1	2.92 $\pm$ 0.45	3.1 $\pm$ 0.4
Non-growing + Oryzalin +auxin	4.3 $\pm$ 0.8	1.9 $\pm$ 0.1	2.70 $\pm$ 0.43	3.7 $\pm$ 0.1

TABLE 2 The tubular dynamics exhibited by the cortical ER tubules

The different tubular dynamics in the cortical ER were defined based on the nature of movement of the tubules that led to their subsequent branching, severing , splitting or fusion. These observations were made in the cortical ER of both treated and untreated epidermal cells. The treated cells include non growing cells treated with 10μM auxin(Non-growing +auxin), 0.1μM latrunculin and 10μM auxin (Non-growing + Latrunculin + auxin), and 20μM oryzalin and 10μM auxin(Non-growing + Oryzalin +auxin). This table is based on visual observation of an area of 250μm^2 over a time frame of 100 seconds in a sample size of (n=10cells) for each of the untreated and treated cells. Since the proportion of cortical ER is different for both treated and untreated cells. The total amount of cortical ER was measured in each of the 250μm^2 area observed for the different tubular movement. The frequency of the occurrence of the movement was divided by the total cortical area present in the 250μm^2 area under observation. Thus the frequency of occurrence of every movement mentioned in the table below is proportional to the total amount of the cortical ER.

Cell type/ treatment	Branch extends and attaches	Branch extends and retracts	Lateral fusion of tubules	Lateral splitting of tubules	Severing and absorption of tubule	Sliding of tubular junction
Non-growing	0.5 ± 0.52	0.8 ± 0.42	0.6 ± 0.51	0.4 ± 0.51	0.7 ± 0.48	4.6 ± 1.51
Growing	1 ± 0.6	0.7 ± 0.48	1.6 ± 0.51	0.7 ± 0.48	0.3 ± 0.48	6.2 ± 4.7
Non-growing +Auxin	1 ± 0.6	0.6 ± 0.51	1.6 ±0.5	0.6 ±0.51	0.4 ± 0.51	8.8 ± 2.82
Non-growing +Latrunculin B + auxin	0.3 ± 0.48	0.1 ± 0.31	0.4 ± 0.51	0.3 ± 0.48	0.1± 0.31	3 ± 1.24
Nongrowing +Oryzalin + auxin	0.4 ± 0.51	0.2 ± 0.42	0.6 ± 0.51	0.2 ± 0.42	0.2 ± 0.42	0

Figure 8. New tubules emerged, extended and coalesced with the existing tubular ER array, thus, splitting an existing polygon into two new polygons

The arrow (a) indicates the point of emergence of the new tubule. The tubule continues to emerge as shown by the arrow (b). The tubule extends across the inter-tubular space (c, d). The tubule has fused with another tubule on the opposite side of the inter-tubular space (e) and, it remains a part of the polygonal ER array (f).
Bar = 5μ

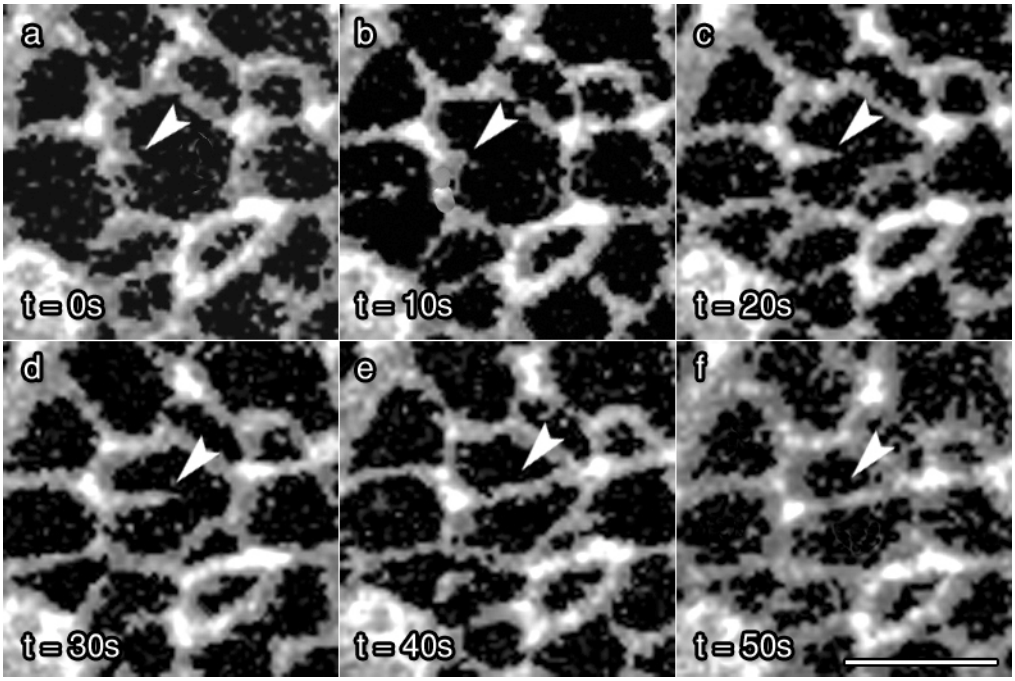
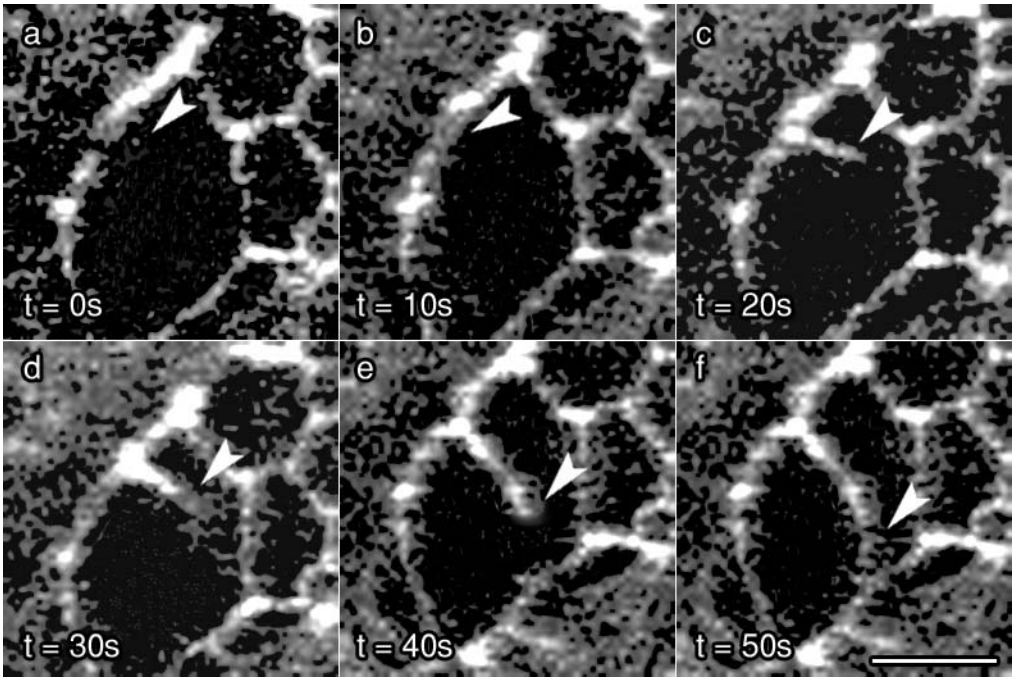

a
t = 0s
b
t = 10s
c
t = 20s
d
t = 30s
e
t = 40s
f
t = 50s

Figure 9. New polygons were also formed by tubular branching in non-growing cells.

The arrows indicate the point of emergence of the new tubule (a, b). The arrows point to the tubule as it extends across the inter-tubular space (c, d, e). The arrow indicates the point of attachment of the new tubule with the existing tubule on the opposite side to form two new polygons (f).

Bar = 5μ


a
t = 0s
b
t = 10s
c
t = 20s
d
t = 30s
e
t = 40s
f
t = 50s

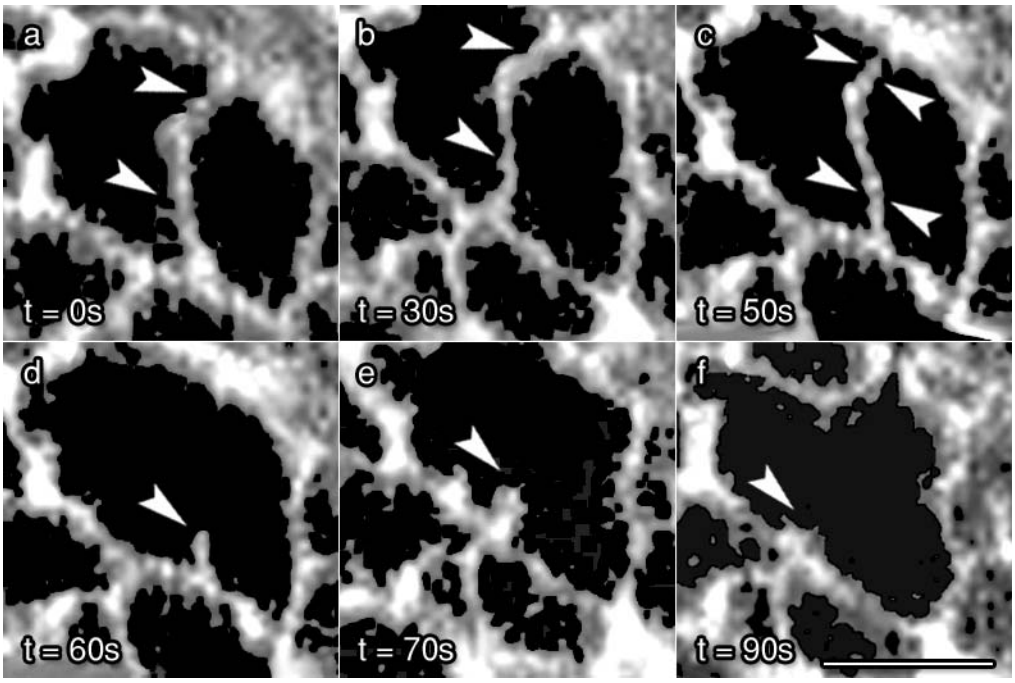
The new tubule did not always fuse with the opposite side of the polygon. Some of the tubules (average 0.7 $\pm$ 0.48 times in a 250μm^2 area in 100 seconds) retracted along their path of origin (Table 2). Tubule branching was not restricted to one branch per polygonal structure. Two or three tubules emerged within the same polygon, although this was rare. The branching tubules always remained in contact with some part of the cortical ER network.

To maintain the equilibrium state of the cortical ER network, the increase in the ER network needs to be balanced by its reduction. Reduction occurred by severing (breaking of the tubule from its point of attachment and gradually shrinking in size until it is reabsorbed into the ER) and subsequent absorption of tubules into the existing array or fusion of parallel ER tubules. Before severing, a tubule became distorted in the region of the break (Fig 10 a). It is not clear if this bend in the tubule was due to an applied force or the loss of previous support that left the tubule sagging (hanging loosely due to loss of support that held it tautly in place). The tubule severed within the distorted region and then became shorter as it gradually retracted into the attached tubule. This breakage followed by retraction resulted in the two polygons reducing to one (Fig 10). These occurred on an average of 0.3 + 0.48 times in a 250μm^2 area over 100 seconds (Table 2). It is important to note that sagging often did not sever. The second method for the reduction of the cortical ER array was lateral sliding and subsequent fusion of tubules (Fig 7). A tubule would slide towards one of the neighbouring parallel tubules (Fig 7 b-c). The length of the sliding tubule became shorter as it was reabsorbed into the attached tubules (Fig7d-e). Eventually, it fused with the parallel tubule reducing two polygons to one
(Fig 7f).

Figure 10. The severing of tubule and its subsequent absorption into the polygonal cortical ER network of a growing epidermal cell

Prior to the severing, the tubule became curved, as if it had lost support or was being pulled out of line (region between arrows in a, b, c). The curved region between the double arrows (c) was lost within 10 seconds (d, e), while, the remainder of the tubule was reabsorbed into the array over 20 seconds (e-f).

Bar = 5μ


a
t = 0s
b
t = 30s
c
t = 50s
d
t = 60s
e
t = 70s
f
t = 90s

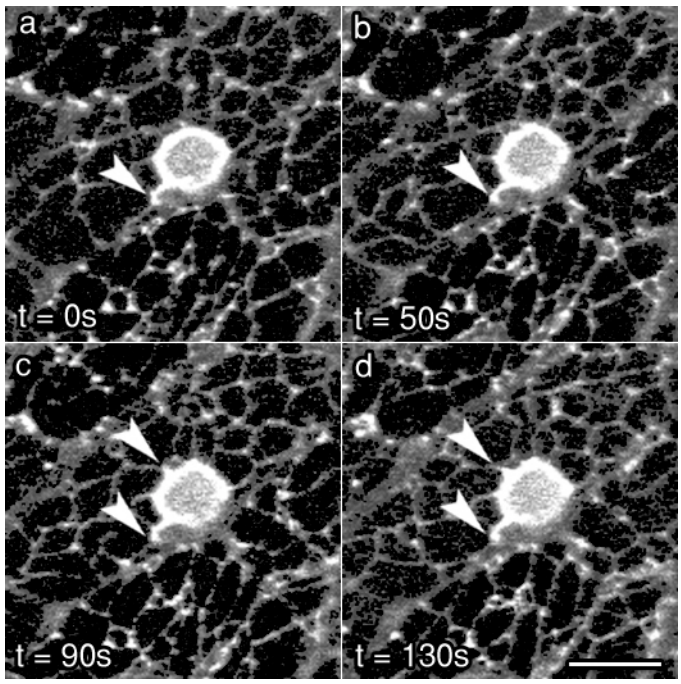
3.2.1 Dynamic equilibrium of the ER network

The dynamic cortical ER maintained an equilibrium state despite the reduction and addition of tubular ER arrays. The overall proportion of the area occupied by the cortical ER remained almost the same during the period of observation. The concomitant reduction and addition of the tubules observed in 250μm^2 area in (n=10 cells) over a period of 60 seconds showed that the total cortical area remained constant. The average area occupied by the cortical ER observed was 50.42 $\pm$ 17.07 μm^2 and after the 60 seconds was found to cover on an average 50.68 $\pm$ 16.29 μm^2 in the same cells (Paired T- test, Appendix 5). The cortical ER had neither a specific zone of tubular addition by branching nor a zone of tubular reduction by severing as these events occurred in all parts of the cortical ER network. Despite these various dynamic movements, there was no net change in the basic structure of the ER network.

In comparison with the tubular ER, the lamellae tended to be static and showed very little individual movement (Fig 5). However, on a few occasions a lamella was observed to slide along a tubule. This appeared to be analogous to the sliding of tubules described above. However, tubules attached to the lamellae were motile and underwent tubular branching and re-absorption into the lamellar sites. In addition, there was not much change in the shapes of the lamellae during these tubular dynamics. The nuclear envelope (n=40 nuclei) did not show much individual movement and tended to stay in the same spot over the duration that it was observed (Fig 5 and Fig11). However, the tubules that connected with the nuclear envelope were motile with tubules branching from, retracting into and sliding along the nuclear envelope (Fig 11).

Figure 11. The tubules attached to the nuclear envelope are dynamic

The nuclear envelope was connected to the cortical ER network by tubules. The tubules pointed by the arrows were dynamic in nature and appeared to be pulled in different directions exerting a slight pull on the nuclear envelope in the direction of the force. This is observed clearly, as the nuclear envelope appeared to be bright because of the concentration of the GFP retained in the ER around it.
Bar = 10μ


a
t = 0s
b
t = 50s
c
t = 90s
d
t = 130s

3.3 The structure and motility of cortical ER changed with cell growth.

The growing and the non-growing cells were different with respect to their cortical ER structure (Fig 12). Growing cells (n=1170 cells) had fewer tubules that were longer and thinner (Table 1) with larger inter- tubular spaces compared to the cells that had ceased to grow (n=1120 cells) (Fig 12). This resulted in large inter-tubular areas and smaller average proportion of cortical cytoplasm occupied by cortical ER in growing cells (Fig 12 b and Fig 13). The lamellae were reduced in size and number in growing cells (Table1). The nuclear envelope was more conspicuous due to its brighter fluorescence in growing cells compared to the non-growing ones (Fig 12 e).

The tubules of the cortical ER network in growing cells and non-growing cells also showed differences in dynamics. Growing cells showed more tubular branching, severing, fusion and splitting per unit area in the non-growing cells (Table 2). Given that there was less ER per unit area in the growing cells, the motility per unit of ER was much greater. Even with their increased frequency of motility, they maintained a dynamic equilibrium as the average proportion of cortical cytoplasm occupied by the ER remained the same from the beginning until the end of observation as explained in the dynamic equilibrium section. When I quantified the total motility by subtracting the binarized images taken 60 seconds apart, I found that more ER had moved in the growing cells as compared to the non-growing cells (Fig 15).

Figure 12. Comparison of the cortical ER and nuclear envelope of auxin treated non-growing cells with untreated non-growing and growing cells

The cortical ER network was different in the non-growing cell (a), the growing cell (b) and auxin-treated cells (c). The difference is shown by the difference in the arrangement of the tubules that make up the network. The other point of difference between these cells is the nuclear envelope that is not very fluorescent in the non-growing cell (d), while, it is more fluorescent in the auxin-treated cell (f) and the most in the growing cell (e).

Bar = 10μ

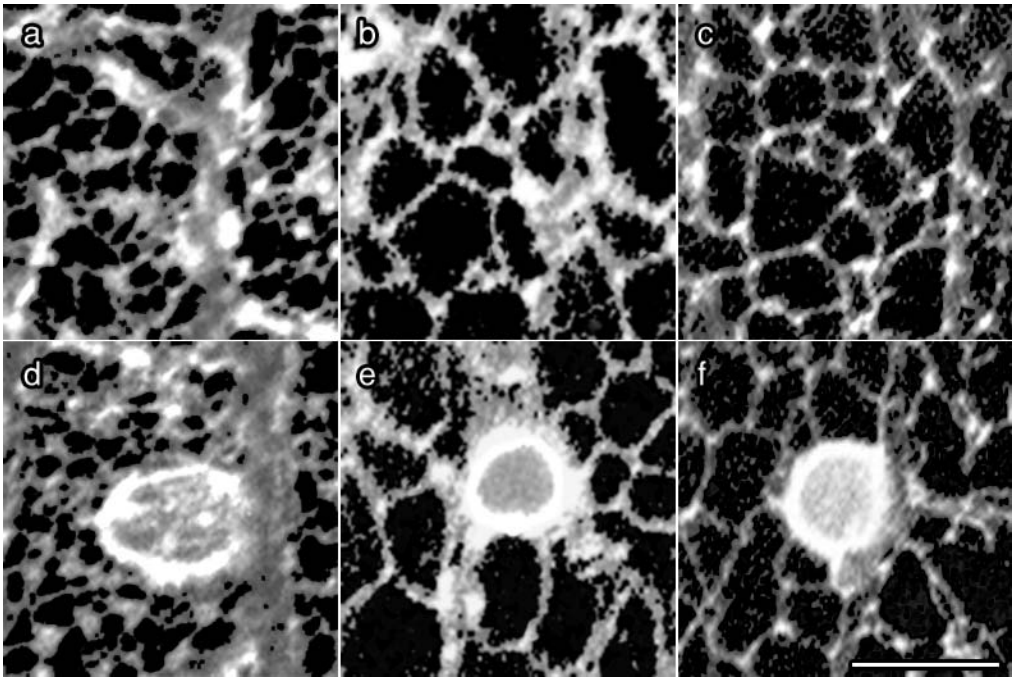

a
b
c
d
e
f

Fig.13 Comparison of the proportion of cortical ER between the growing and non-growing cells with the auxin treated non-growing cells. Note that the non- growing, auxin treated cells had resumed growth.

The cortical ER of growing (Growing) and Non-growing (Nongrow) cells was compared with non-growing cells treated with 10μM auxin (Auxin). The proportion of the cortical ER was measured using the ImagePro software from the confocal microscope images of the different treated and untreated cells. The sample size of (n=10 cells) was used for each group. Since the cell sizes varied comparison is based on the relative proportions.

ANOVA (P=0, Critical Q Value= 3.031). Tukey test showed growing and auxin treated cells to group together, while the non-growing cells formed their own group

Error bar is standard deviation

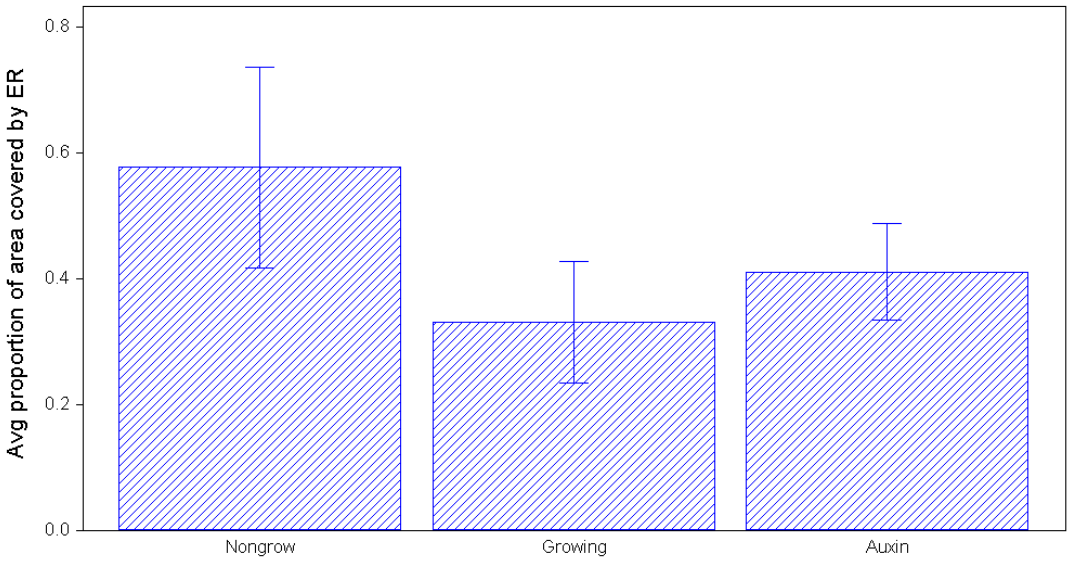

Avg proportion of area covered by ER
0.8
0.6
0.4
0.2
0.0
Nongrow
Growing
Auxin

Fig.14 Comparison of the proportion of the cortical ER occupied in the growing, non-growing and treated non-growing cells

The proportion of cortical ER in untreated growing (Growing) and Non-growing (Nongrow) cells was compared with non-growing cells treated with 10μM auxin (Auxin), 0.1μM latrunculin and 10μM auxin (Latraux) and 20μM oryzalin and 10μM auxin (Oryaux). The sample size was 10 cells for both treated and untreated cells. The images of the different groups of cells were obtained from the confocal microscope images. The amount of cortical ER covered in the cells was measured using the ImagePro software and converted to graph using the Statistix version 8 analytical software. Since the cell sizes and the ER amount varied comparison of cortical ER was based on its relative proportions in the cell.

ANOVA (P= 0, Critical Q Value= 4.019).

Tukey test showed that the growing, auxin, and latrunculin and auxin treated samples were not different from one another. They were different from non-growing that formed separate group. While oryzalin and auxin overlapped between the other two groups.

Error bar is standard deviation

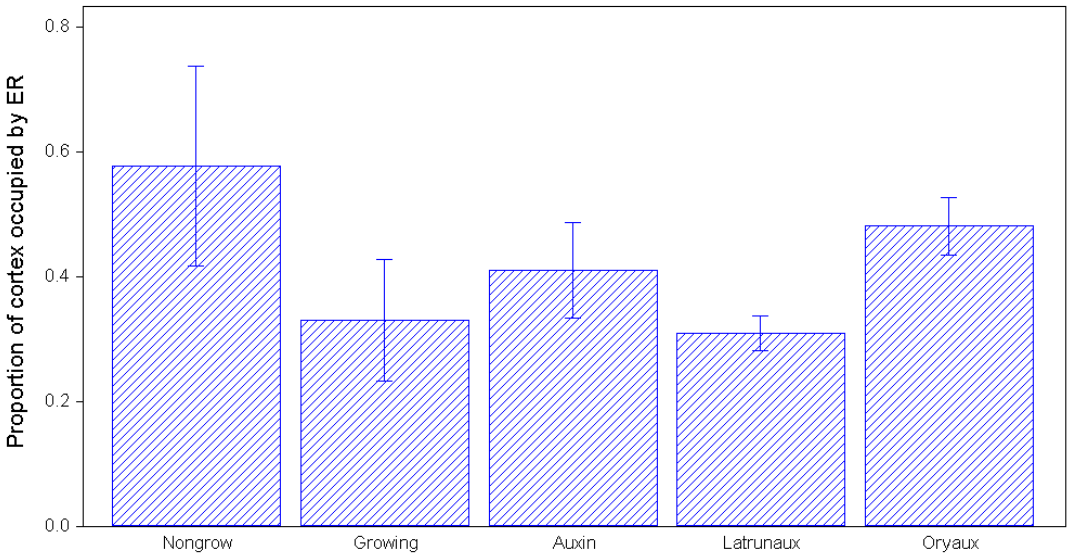
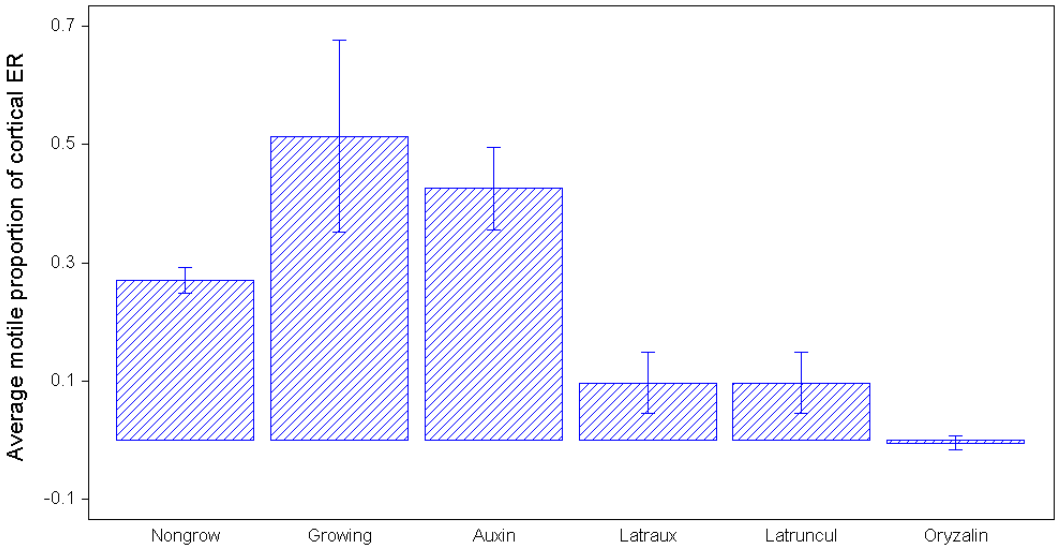

Proportion of cortex occupied by ER
0.8
0.6
0.4
0.2
0.0
Nongrow
Growing
Auxin
Latrunaux
Oryaux

Fig. 15. Comparison of the average motile proportion of cortical ER in both treated and untreated cells over 60 seconds time frame.

The average motile parts of the untreated growing cells (Growing) and non-growing (Nongrow) cells are compared with the average motile parts of the non-growing cells treated with 10μM auxin (Auxin), 0.1μM latrunculin (Latruncul), 0.1μM latrunculin and 10μM auxin(Latraux) and 20μM oryzalin (Oryzalin). The sample size was 10 (cells) for both treated and untreated cells. The observation time was 60 seconds and since the cell size differed, a relative proportion of cortical ER was used for the calculations. Two sets of images were selected from a time series of confocal microscopy that were 60 seconds apart. The images were binarized using Metamorph software. The first image from each set was assumed as (stationary) and the second image taken after 60 seconds in the series was taken as the (motility images). The motility images were subtracted from the stationary images and (resultant images) were obtained. These resultant images showed the parts of the cortical ER that were not motile. These resultant images were subtracted from the first set of images to obtain the motile parts of the cortical ER. The area covered by motile ER was measured using the ImagePro software and converted to a graph using the Statistix version 8 analytical software.

ANOVA (P=0, Critical Q Value = 4.307)
Tukey test showed that the growing and auxin samples were not different from each other. The non-growing cell forms a separate group. The cortical ER of the oryzalin and auxin sample disintegrated when exposed to the laser. Hence, data is not given here. See text for results.

Error bar is standard deviation


Average motile proportion of cortical ER
0.7
0.5
0.3
0.1
-0.1
Nongrow
Growing
Auxin
Latraux
Latruncul
Oryzalin

3.4 Auxin induced growth of non- growing epidermal cells

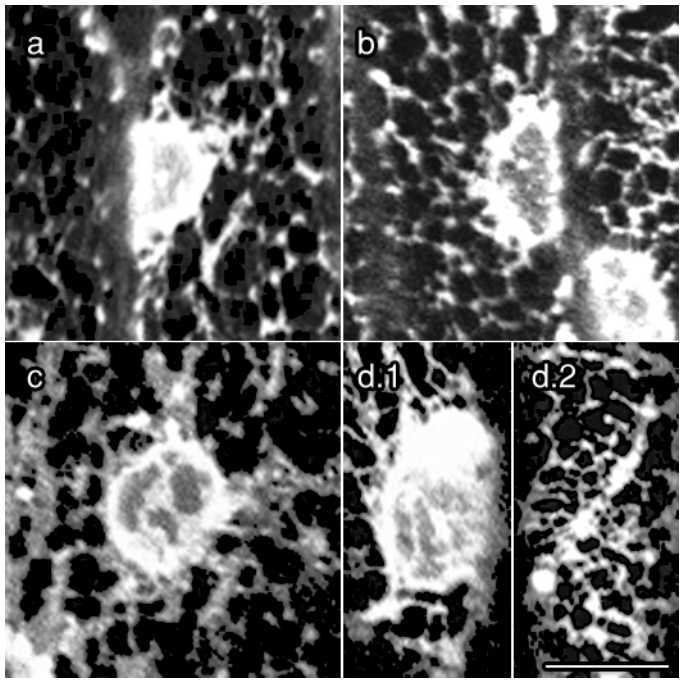
Non-growing cells (n= 10) were treated with 10μM Auxin (NAA) for 24 hours and the change in cell size was measured. It was found that the auxin treated cells had enlarged as cell length increased from an average of 7.3 $\pm$ 1.63 μm to 12.04 $\pm$ 1.13μm (Appendix 6). This showed as expected (Syndonia, 1992) an exogenous application of auxin-induced growth in epidermal cells of pea tendril. I observed auxin treated to test the effect of this induced growth on the cortical ER structure and dynamics. The auxin assay was coupled with cytoskeleton disruption to determine the role of the cytoskeleton in this change.

3.5 Auxin treated cells underwent changes in ER structure and motility

Non-growing epidermal cells (n=210 cells) were treated with 10μM auxin for 30 minutes. In response to this, the ER network changed and resembled the growing cell more than the non-growing cell (Fig 12 c) in structure. The cortical ER network of the treated cells had become less dense and its structure resembled that of the growing cell (Fig 12). I also observed reduced thickness of the tubules, increased tubule length along with fewer and smaller lamellae (Table 1). The nuclear envelope fluoresced more brightly compared to that of the non-growing cell (Fig 12 c). In addition, there were increased tubular dynamics (Table 2). The quantitative analysis of the images obtained from the auxin treated non-growing cells showed that the area covered by the cortical ER network was less than the area covered in non-growing cells (Fig 13). Consequently, there was an increase in inter- tubular area. There was also an increase in the overall tubular motility as compared to the non-growing cells (Fig 15).

Figure 16. The change in cortical ER structure in response to microtubule disruption using oryzalin.

On addition of (a) 10μM oryzalin to the non-growing cell, a change in the distribution of the cortical ER was observed. It seemed as though the ER began to move in to the nuclear envelope. A similar observation was made on addition using 20μM oryzalin and 10μM auxin (b), but there was a recovery in the structure after 24 hours, when the drug was washed (d1and d2). However, on addition of 50μM oryzalin there was an increase in the flattened sheet-like lamellar-like ER that spread throughout the cortex. (c) consequently, the polygonal array structure was lost which could not be recovered despite washing out the drug.
Bar = 10μ


a
b
c
d.1
d.2

Thus, induced growth changed the structure and motility of the ER network in the non-growing epidermal cells (Fig 12, Fig 13 and Fig15).

3.6 Disruption of the microtubules

Here, I have disrupted the microtubules with the inhibitor oryzalin and actin microfilaments with latrunculin B in order to determine whether the auxin induced change in the ER structure and the dynamics of non- growing cells was dependent on the intact cytoskeleton.

The non-growing cells were treated with 20µM oryzalin in the absence of auxin. Even though, the cortical ER remained intact, it showed reduced motility (Fig 15). The tubule length and lamellar number did not change. There was increased fluorescence of the nuclear envelope (Fig 16 a). The ER appeared to have collapsed in the nuclear envelope. Non-growing cells treated with a high concentration of oryzalin (50µM) showed disruption of the cortical ER lattice (Fig 16 c). The bright nuclear envelope implied that some ER had retracted into the envelope. However, this fluorescence was patchy in nature that was atypical of nuclear envelope in treated cells. Pieces of ER were scattered throughout the cortical region indicating that the cortical ER had broken apart (Fig 16 c). Cells treated this way did not recover a normal ER array structurally even after 24 hours of the removal of oryzalin.

When non-growing cells were treated with (20µM) oryzalin in the presence of (10µM) auxin, the cortical ER did not become like that of a growing cell (Fig 16 b). While the network did alter its structure slightly, the inter-tubular area remained

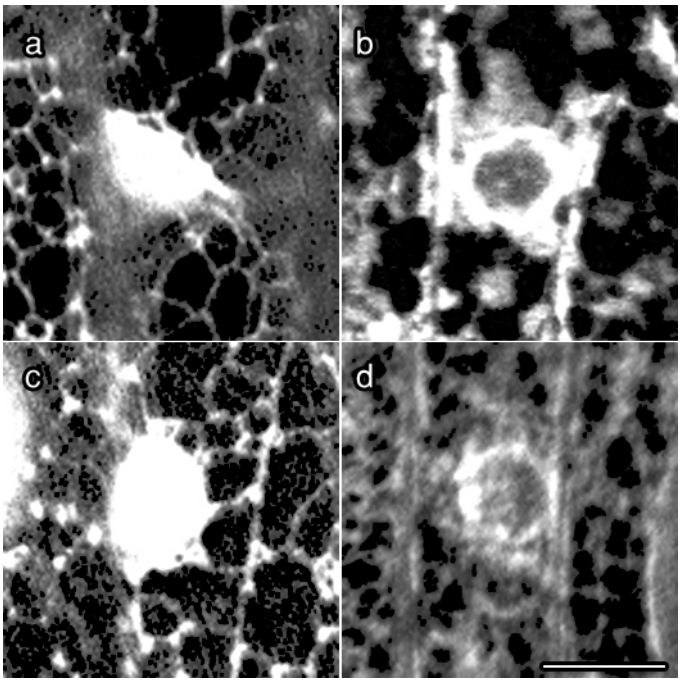
small and did not become as large as seen in cells treated with only auxin. Also, while the number of lamella reduced and the nuclear envelope increased in fluorescence, neither of these were to the extent observed in the untreated growing or auxin treated cells (Fig 16 b). Furthermore, motility results were hampered by the instability of cortical ER in the auxin and oryzalin-treated cells (Fig 15). During observation with the confocal laser microscope, the cortical ER network began to disassemble. At the end of 10 scans, 10 seconds apart a large part of the ER lattice was lost. During this, ER was absorbed into the nuclear envelope and it broke up into isolated islands. This resulted in a change in ER distribution that was not related to the movement of the ER hence, the data is not represented in the graph (Fig 15). Cells regained normal ER structure and dynamics following wash out of the oryzalin and auxin over duration of 24 hours (Fig 16 d1, d2)

3.7 Disrupting actin microfilaments caused tubules to be lost

On treatment of the non-growing cells (n=35 cells) with 0.1μM of latrunculin B, there was a change in the structure of the ER network and it resembled the network of a growing or auxin treated cell (Fig17 a). While the ER structure was similar to the growing cell, the dynamics were not. Tubular motility was greatly reduced in the latrunculin B treated cells (Fig 15). When the non-growing cells (n=28 cells) were treated with high concentration of latrunculin B (1μM), it perturbed the structure of the array (Fig 17 b). Although a lattice with large intra-tubular space developed. The lattice was formed from flattened lamellar like pieces of ER instead of the tubules typical of the growing cells (Fig 17). The nuclear envelope appeared to have the same patches of lamella- like ER around it. This atypical network showed little movement.

Figure 17. The change in cortical ER structure in response to actin microfilament disruption using latrunculin B

Addition of 0.1μM of latrunculin B to non growing cells, made the ER appear similar to that observed in the growing cells with reduced tubular density ,but it had more ER in the nuclear envelope (a). Addition of higher concentration of 1μM latrunculin B made the ER look atypical with patches of ER attached to the nuclear envelope and at various sites in the ER (b). The addition of 0.1μM latrunculin B and 10μM auxin, also made the ER appear similar to that observed in the low concentration treatments of latrunculin B. On washing away the latrunculin B and auxin for 24 hours, the ER appeared to recover its original structure of the non-growing cells (d).
Bar = 10μ


a
b
c
d

The cortical ER lattice showed its normal response to 10µM, auxin treatment of non-growing cells when 0.1µm latrunculin B was present (n=58 cells), with enlarged inter-tubular spaces (Fig 17 c), reduced number of lamellae (Table1) an increased fluorescence of the nuclear envelope (Fig 17 c), and the increase in level of tubular dynamics (Table 2). The changes in the ER structure were similar to those found in growing and auxin treated cells. Cells recovered normal ER structure and motility once the latrunculin B and auxin were removed (Fig 17 d). However, motility again was greatly reduced (Fig 15).

CHAPTER 4

Discussion

4.1 The structure of the cortical ER was best described as a dynamic equilibrium

The cortical ER was an inter-connected lattice of tubules and lamellae. The tubular ER was mobile and increased in distribution by constantly branching, splitting and extending to form new tubules. Concomitantly, there was a reduction in tubules by severing and retracting. The resultant effect of all this tubular movement was that the ER network continually changed its structure and appeared to have a slackening and tightening of the network at various sites of reduction and expansion respectively. However since the net result was the maintenance of the same amount of ER, I have described it as a dynamic equilibrium. While other research groups have reported an overall dynamic nature of the network (Quader *et al*., 1987; Boevink *et al.,* 1998; Ridge *et al.,* 1999 and Samaj *et al.*, 2000), I am the first to provide evidence that it behaves as a dynamic equilibrium.

The structure and dynamics of the cortical ER of the epidermal cells of the pea tendril was typical of other epidermal cells including those of the onion bulb (Quader *et al.*,1987 and Knebel *et al.*, 1990), the *Arabidopsis* root (Ridge *et al.,* 1999) and in the maize root (Samaj *et al*.,2000) (Chapter 3, Fig 4).

I did not report the observation of any fusiform bodies like those reported in other studies (Haseloff *et al.*, 1997 and Robert Ridge *et al.,* 1999). These bodies have only been reported in *Arabidopsis spp* and not in other GFP-labelled plants like tobacco

(Boevink *et al*., 1996-1998). It is hypothesised that these are specific to *brassicaceae* plant species (Matsushima *et al.*, 2003).

I did not notice any pattern of distribution for the lamellae in any of the cells observed. In onion cells, lamellae were distributed in rows helicoidally to the longer axis of the cells (Quader *et al.*, 1987).

4.2 The growing cells had different ER structure and dynamics

When the epidermal cells were growing, there was a shift in the dynamic equilibrium accompanied by the change in structure of the cortical ER. Growing cells had less ER per unit area of the cortical cytoplasm that consequently formed a more open lattice compared to the non-growing cells (Chapter 3, Fig.12 b). There was a shift to a higher proportion of the ER in the tubular form with very few lamellae (Chapter 3, Table 1). Concomitant with the increase in the tubular ER, there was an increase in the tubular associated dynamics. The difference in tubular dynamics of the cortical ER in growing and non-growing cells has been reported for the first time in my study. I have quantified the dynamics and on comparison found that the growing cells showed more tubular dynamics than the non- growing cells (Chapter 3, Fig 15). Although the dynamics in both growing and non-growing cells have been reported (Ridge *et al*., 1999), there have been no reports on the difference in the motility rates (rate of movement of ER per unit area).

Addition of auxin to the non-growing cells induced the cells to resume growth and changed the cortical ER structure and motility to that of a typical growing cell.

This provides evidence that shift seen in the dynamic equilibrium is related to growth. I was the first to observe this change in the ER structure of non-growing epidermal cells in response to induced growth. However, I am not the first to observe a difference in the ER structure between growing and non-growing cells (Ridge *et al.*, 1999). Ridge *et al*., (1999), reported a change in ER structure associated with the growth of the *Arabidopsis* root epidermal cells but the results of this study were in contrast to my observations. ER in the growing cells covered more of the cortical cytoplasm, and was in the form of perforated sheets as opposed to the open tubular array that I saw in growing pea cells. This could be because my study involved the epidermal cells in the tendril that were back from the meristem and presumably growing at a slower rate. The study of Ridge and co-workers (1999) observed rapidly growing root cells adjacent to the meristem. It may well be that slow and rapidly growing cells have different ER structures. Alternatively, the sheets of cortical ER reported by (Ridge *et al*., 1999) may be special to expanding root cells.

Interestingly, they also noted that the tip-growing root hair had a third type of ER structure, indicating that the change in ER structure is not only related to the presence or absence of growth but that the mode of growth is also important. Here, I described cells growing within intercalary made of cell wall deposition.

Studies in the past have reported the presence of higher amount of cortical ER in certain specialized cells capable of producing auxin as in *Vinca* and *Acacia* (J.Lipetz, 1967-1970) and also reported that presence of auxin changed the ER architecture in growing cells (Stickens, 1996). However, my study is the first to provide evidence

that the exogenous application of auxin not only changed the structure of the cortical ER in non-growing epidermal cells but also increased its tubular dynamics.

4.3 Perturbation of the cytoskeleton changed the cortical ER lattice

The change in the ER structure and dynamics associated with growth may involve both actin microfilaments and the microtubules. When I disrupted the microtubules using oryzalin, I found that even low concentration of the drug (10μM) inhibited the motility of the ER tubules. Besides oryzalin is a plant herbicide that binds reversibly to the plant microtubule (Hugdahl and Morejohn, 1993) and depolymerises the microtubules by binding to them (Morejohn *et al*., 1987). Waterman-Storer *et al*., 1997, have demonstrated the movement of the ER tubules along the microtubules and their attachment to their growing end. There have been reports that the actin microfilaments and microtubules interact together in plant cells to perform the function of supporting the ER structure (Kobayashi *et al.*, 1988; Collings *et al*., 1998; Takesue and Shibaoka 1998 and Samaj *et al.*, 2000), while in some studies only actin interaction has been proposed (Quader *et al.*, 1989). In this study, it was reported that the tubules recovered in the presence of microtubule disrupting drugs and not the actin disrupting drugs, the cold shock treatment used to stop the ER dynamics could have inhibitory effects on the cytoplasm as well. In addition, they used very low concentrations of the oryzalin drug to inhibit microtubules.

In my study, perturbing microtubules did cause the ER to change; these alterations were rarely consistent with those associated with growing cells. Instead, it seemed that loss of microtubules leads to the breakdown of the cortical lattice. When the network remained intact, it looked atypical and lacked motility (Chapter 3, Fig 16).

When the actin microfilaments were perturbed in the non-growing cells by the addition of low concentration (0.1μM) of latrunculin B, the ER network switched to be like that observed in a growing cell (Chapter 3, Fig 17 a). The network was motile though much less in comparison to the growing and growth induced non-growing cells (Table 2). This was not the case on addition of higher concentration (1μM) of latrunculin B, which resulted in the complete breakdown of the ER structure and its accumulation around the nuclear envelope and other random sites throughout the cortical ER (Chapter 3, Fig 17 b). Addition of auxin along with this actin inhibitor, latrunculin B, to non-growing cells gave similar results. The tubular arrays resembled the growing and the auxin induced cells structural arrangement, moreover tubular dynamics were reduced (Chapter 3, Table 2). One probable reason for this change could be the breakdown of actin from denser filaments F-actin to G-actin (globular actin or monomers of F-actin) using latrunculin B in congruence with previous researches (Yarmola *et al.*, 2000). This shift in the equilibrium between actin microfilaments and G-actin caused a change in the structure and dynamics of the ER network. Implying that, actin microfilaments maintain the lamellae and the tubular ER lattice. The reduced motility associated with loss of F-actin indicates that actin may be required for normal motility of the ER.

My observations of the difference in the ER structure between growing and non-growing cells complements the reported difference in actin structure of growing and non-growing cells of maize coleoptiles (Waller and Nick, 1997). This study reported loosely arranged F-actin network in growing cells and denser fused network in non-growing cells. This arrangement of the actin network is similar to the arrangement of tubules of the cortical ER in the growing and non-growing cells in my study.

However, this study did not show the co-localization of the actin network with the cortical ER network, making it difficult to draw any co-relation between the actin filaments and cortical ER based on this study. This relatively new topic needs further exploration in order to determine how the difference in arrangement of the actin cytoskeleton correlates to the different stages of cell growth.

In this work, I have assumed that oryzalin and latrunculin B disrupt the cytoskeleton. This may be risky, as such inhibitors may not have had the expected effect and their are studies that show that use of such drugs can have indirect effects on other components of the cytoskeleton (Kobayashi *et al.*,1988, Collings *et al.*,1998 and Takesue and Shibaoka,1998). However, since other reports using similar concentrations of drugs were able to show disruption of the microtubules (Sieberer *et al.*, 2002) or actin filaments (Mathur *et al.*, 1999 and Baluska *et al.*, 2000b), it supports the similar use of the inhibitors here. The microtubules and actin network have not been studied in the epidermal cells of pea tendril. Hence, a logical extension of my work would be to observe the cytoskeleton and its disruption with the inhibitors.

The second assumption was that the actin microfilament and microtubules networks were independent in their involvement with respect to the distribution of ER. This assumption was based on the ER studies, which showed that either the actin microfilaments (Quader *et al.*, 1987) or the microtubules (Terasaki *et al.*, 1986) were involved. However, other studies (Terasaki *et al.*, 1986; Samaj *et al.*, 2000) reported the interactions of both actin and microtubules. This means that a study of actin and microtubules would be required in pea tendril epidermal cells.

In interpreting my results, I assumed that latrunculin B perturbed only actin microfilaments and the microtubules remained intact. Similarly, it was assumed that the treatment of the cells with oryzalin left actin microfilaments intact. These assumptions were based on the study conducted by (Quader *et al.*, 1989) that demonstrated that the actin microfilaments remained intact while the microtubules were disrupted using oryzalin.

Unfortunately, sometimes the tools used in the study can also perturb the cells, for example, the toxic side effects of UV light and photobleaching of $DIOC_6(3)$(Chapter 1, section 1.4.1). Since GFP is resilient to photobleaching, it has been assumed that observing live cells labelled with GFP using low doses of light (as is the case with confocal laser scanning microscopy) would have minimal impact on the health of the observed cells. Other observations of GFP-labelled ER have failed to report any adverse side effects supporting this idea (Haseloff *et al.*, 1997 and Ridge *et al.*, 1999). I found that most cells were stable when observed, but the non-growing cells treated with oryzalin and auxin together underwent disintegration during the observation. Each successive laser scan resulted in additional damage. This showed that either energy from the laser or free radicals released during photo bleach do have an adverse effect on the cells. However, when the microtubules were intact, the cells can cope with this observation-induced stress.

The fact that the structure of ER did not change, and the same proportion of the cortex was covered with ER at the start and at the end of the observations, supports my claim

that the observation regime I used (1 scan every 10 seconds) did not unduly perturb the cells.

4.4 The Nuclear envelope may be an ER organizing centre

The nuclear envelope is inter-connected with the cortical ER array. It may well be the focal point for the ER organization. For example, when the cortical array was reduced in growing cells, or auxin treated cells, there appeared to be more ER around the nucleus implying that the reabsorbed ER had been retracted into the nuclear envelope instead of being degraded (Chapter 3, Fig12). This implied that the nuclear envelope might be a focal point for the organization of the ER and act as an ER organizing centre.

The high level of fluorescence of the nuclear membrane in growing cells compared to non-growing cells can be related to the amount of brightness in the nuclear envelope that was directly proportional to the amount of GFP. This assumption was based on the study that reported that the quantity of protein content in the various plant tissues samples was linearly proportional to the GFP fluorescence (Halfhill *et al.*, 2003). In another study, an increase in nuclear volume followed by an increase in the distribution of the ER-localised GFP was reported (Haseloff, 1997). Hence, the level of GFP-fluorescence can be related to the amount of GFP in the nuclear envelope using the degree of fluorescence as an indicator.

4.5 Conclusion

I found that the structure and dynamics of the cortical ER was different in growing and non-growing epidermal cells, and that both the actin and the microtubule cytoskeleton networks were likely to play a role in determining these features of the array. I reported for the first time that auxin-induced growth could change the ER structure and motility.

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APPENDIX 1

Preparation of pea growth media

Media preparation

STOCK solns	**STOCK conc**	**Add**
MS Macro	20x	50ml
MS Micro	200x	5ml
MS Iron	200x	5ml
B5 Vits	200x	5ml
Bovine albumin (1.3mg/litre)	1mg/ml	1.3ml
Sucrose	30g/litre	30gm
Final volume		make up 1litre
Final pH	5.8	
Agar:Difco	7g/litre	7 gms

MS MACRO STOCK SOLN 20X
Makes 1000mls of 20x stock soln .Start with 900 mls water

	Final	**Add**
NH_4NO_3	412.5mg	33.0gms
KNO_3	475.0mg	38.0gms
$Cacl_2.H_2O$	110.0mg	8.8gms
KH_4PO_4	42.5mg	3.4gms
$MgSO_4.7H_20$	92.5mg	7.4gms

Add water to make upto 1litre

MICRO STOCK SOLN
200x
Makes 1000mls of 200x micro stock.Start with 600 mls of water

	Final	**Add**
H_3BO_3	6.2mg	1.24gms
$MnSO_4.7H_2O$	22.3mg	4.46gm
$ZnSO_47H_2O$	8.6mg	1.72gms
KI(Potassium iodide)	0.83mg	1.66gms
$Na_2MoO_4.2H_2O$	0.25mg	0.05gm
$CoCl_2.2H_20$	0.025mg	0.005gm
$CuSO_4.5H_2O$	0.025mg	0.05gm

Add water to make upto 1 litre

B5 Vitamin Stock 200x
Makes500mls of 200x stock

Initials	**Final conc**	**Add**
Myo-inositol	100mg	10gm
Nicotinic acid	1mg	0.1gm
Pyridoxine HCl	1mg	0.1gm
Thiamine HCl	10mg	1gm

Add water to make upto 500mls

APPENDIX 2

Statistical analysis (ANOVA and Tukey Test) Comparison of the proportion of cortical ER between the growing and non-growing cells with the auxin treated non-growing cells

The data was collated in excel sheets using Microsoft Office Excel 2003 and subjected to statistical analysis using Statistix 8. The ANOVA was one-way because it was doing a simple comparison of the only one factor that was the amount of area covered by the cortical ER network in treated and untreated cells. The Tukey statistical test was also performed which is a multiple comparison test that uses multiple T tests, to examine the differences between all possible pairs of the means in the data (Zar, 1984).

The result of the examples is given in the tables below.

Table A.2.1 ANOVA for area covered by cortical ER at α = 0.05

SUMMARY

Groups	Count	Sum	Mean	SD	Variance
Non-growing	10	5.77	0.577	0.1596	0.0255
Auxin	10	4.11	0.411	0.0767	5.88E-03
Growing	10	3.13	0.313	0.0973	9.48E-03

ANNOVA α = 0.05

Source of variation	SS	DF	MS	F	P-value
Between groups	0.31491	2	0.15745	11.6	0.0002
Within groups	0.36739	27	0.01361		
Total	0.6823	29			

Although the area covered by the cortical ER did not differ between the cells at a significant level of 0.05. It is necessary to perform the Tukey Test to determine where the difference lies.

TABLE A.2.2

The Tukey Test for the difference in the area covered by the cortical ER between the Non-growing, growing and auxin treated cells $\alpha = 0.05$

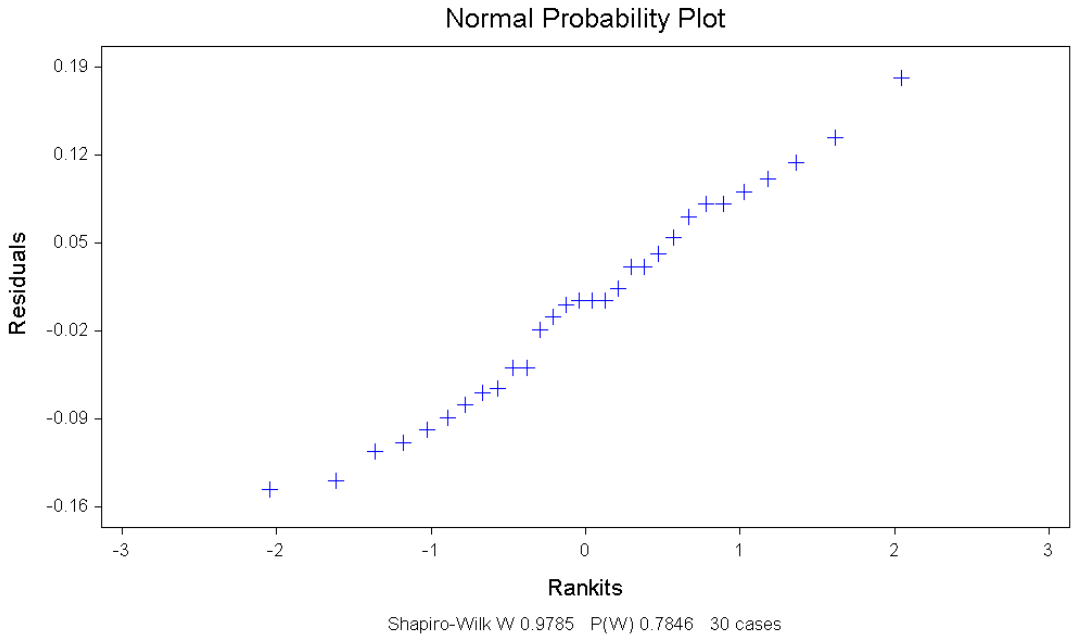
Tukey HSD All-Pairwise Comparisons Test

Variable	Mean	Homogeneous Groups
Nongrowin	0.577	A
Auxin	0.411	B
Growing	0.331	B

Standard Error for Comparison = 0.0522
Critical Value for Comparison = 0.1118
Critical Q Value= 3.031

The Tukey test shows that the growing and auxin treated cells grouped under one common group and the non-growing cells are in a separate group.

Conclusion: The areas covered by the cortical ER in the growing and auxin treated cells nearly the same while that covered in the non-growing cell was different or more.



The Normal Probability Plot conforms to a normal distribution of the variables

APPENDIX 3

Statistical analysis (ANOVA and Tukey Test) for the area of cortex covered by the cortical ER in the treated and untreated cells

The data was collated in excel sheets using Microsoft Office Excel 2003 and subjected to statistical analysis using Statistix 8. The ANOVA was one-way because it was doing a simple comparison of the only one factor that was the amount of area covered by the cortical ER network in treated and untreated cells. The Tukey statistical test was also performed which is a multiple comparison test, that uses multiple T tests, to examine the differences between all possible pairs of the means in the data (Zar,1984).

The results of the examples are given in the tables below.

Table A.3.1 ANOVA for area covered by cortical ER at α = 0.05

SUMMARY

Groups	Count	Sum	Mean	SD	Variance
Latrunculin B + auxin	10	3.1000	0.3100	0.0279	04
Auxin	10	4.1100	0.4110	0.0767	5.877E-03
Growing	10	3.3100	0.3310	0.0973	9.477E-03
Non- growing	10	5.7700	0.5770	0.1596	0.0255
Oryzalin + auxin	10	4.8100	0.4810	0.0461	2.121E-03

ANNOVA α = 0.05

Source of variation	SS	DF	MS	F	P-value
Between groups	0.48452	4	0.12113	13.9	0
Within groups	0.39348	45	0.00874		
Total	0.87800	49			

The area covered by the cortical ER differs between the cells at a significant level of 0.05. It is necessary to perform the Tukey Test to determine where the difference lies.

TABLE A.3.2

The Tukey Test for the difference in the area covered by the cortical ER between the non-growing, growing, auxin, auxin and latrunculin B,auxin and oryzalin treated cells α = 0.05

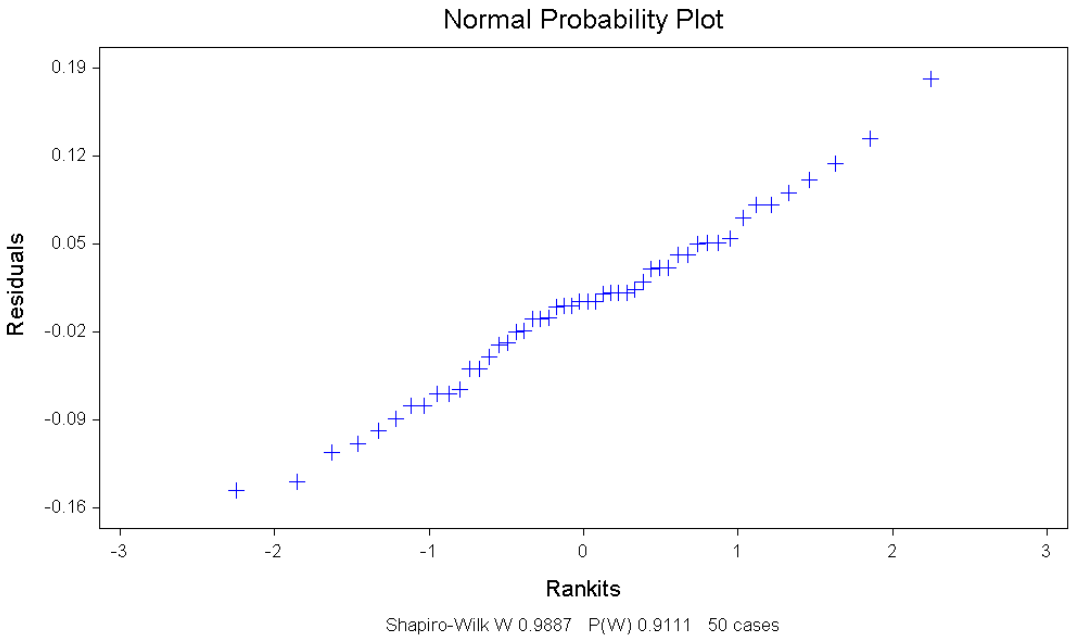
Tukey HSD All-Pairwise Comparisons Test

Variable	Mean	Homogeneous Groups
Non- growing	0.5770	A
Oryzalin + auxin	0.4810	AB
Auxin	0.4110	BC
Growing	0.3310	C
Latrunculin B + auxin	0.3100	C

Standard Error for Comparison = 0.0418
Critical Value for Comparison = 0.1188
Critical Q Value= 4.019

The Tukey test shows that there is an overlap between the non-growing cells and the oryzalin and auxin treated cells also there is an overlap between the growing cells, the auxin treated cells and the latrunculin B and auxin treated cells.

Conclusion: The areas covered by the cortical ER in the growing, auxin, auxin and latrunculinB, auxin and oryzalin treated cells was nearly the same while that covered in the non-growing cell was different or more.



The Normal Probability Plot conforms to a normal distribution of the variables

APPENDIX 4

Statistical analysis (ANOVA and Tukey Test) of the average motile proportion of cortical ER in both treated and untreated cells over 60 seconds time frame

The data was collated in excel sheets using Microsoft Office Excel 2003 and subjected to statistical analysis using Statistix 8. The ANOVA was one-way because it was doing a simple comparison of the only one factor that was the amount of motile cortical ER network in treated and untreated cells. The Tukey statistical test was also performed which is a multiple comparison test, that uses multiple T tests, to examine the differences between all possible pairs of the means in the data (Zar,1984).
The results of the examples are given in the tables below.

Table A.4.1 ANOVA for area covered by cortical ER at α = 0.05

SUMMARY

Groups	Count	Sum	Mean	SD	Variance
Non-growing	10	2.7023	0.2702	0.0217	04
Auxin	10	4.2579	0.4258	0.0699	4.887E-03
Growing	10	5.1390	0.5139	0.1629	0.0265
Latrunculin B +auxin	10	0.9636	0.0964	0.0518	2.679E-03
Latrunculin B	10	0.9636	0.0964	0.0518	2.679E-03
Oryzalin	10	-0.0485	-4.853E-03	0.0122	1.490E-04
Oryzalin + auxin	10	3.5391	0.3539	0.0955	9.122E-03

ANNOVA α = 0.05

Source of variation	SS	DF	MS	F	P-value
Between groups	2.23913	6	0.37319	56.1	0.0000
Within groups	0.41880	63	0.00665		
Total	2.65793	69			

The area covered by the cortical ER differs between the cells at a significant level of 0.05. It is necessary to perform the Tukey Test to determine where the difference lies.

TABLE A.4.2

The Tukey Test for the difference in the area covered by the cortical ER between the Non-growing, growing and auxin treated cells α = 0.05

Tukey HSD All-Pairwise Comparisons Test

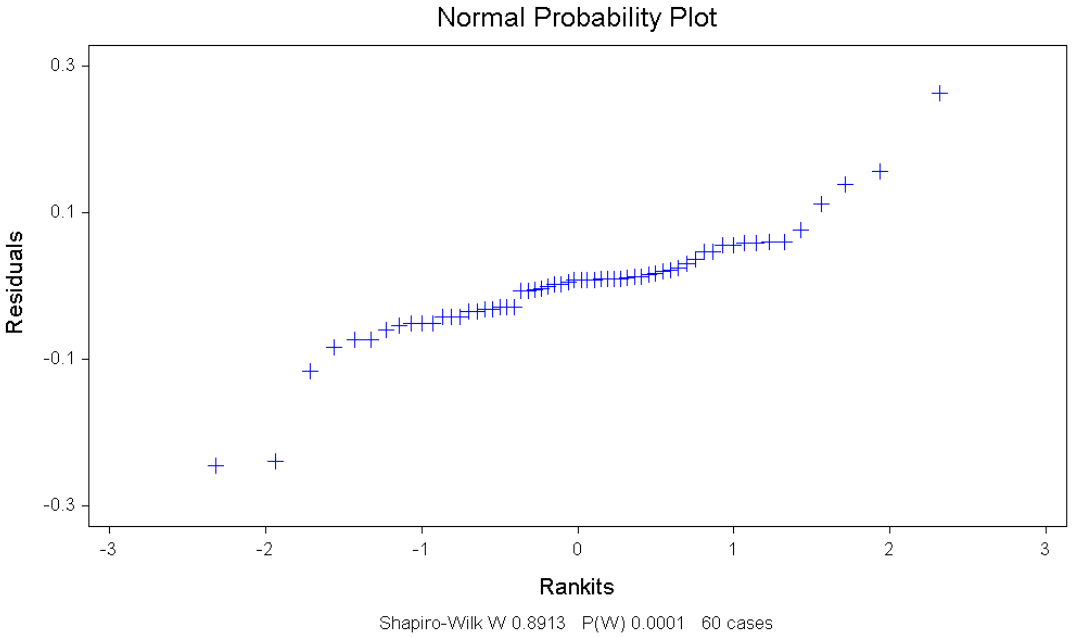
Variable	Mean	Homogeneous Groups
Non- growing	0.2702	A
Auxin	0.4258	B
Growing	0.5139	BC
Latrunculin B+Auxin	0.0964	C
Latrunculin B	0.0964	D
Oryzalin	-4.85E-03	D
Oryzalin + Auxin	0.3539	D

Standard Error for Comparison = 0.0365
Critical Value for Comparison = 0.1110
Critical Q Value= 4.307

The Tukey test shows that the non-growing cells are grouped separately showing that the motility observed was less. The growing and auxin treated cells are overlapping groups also auxin group is overlapping with auxin and latrunculin B treated cells. The latrunculin B , oryzalin and oryzalin and auxin are under one common group.

Conclusion: There are more motile parts in the non-growing cells compared to the

growing, auxin and auxin and latrunculin treated cells. There are not many motile parts of the cortical ER in the latrunculin, oryzalin and oryzalin and auxin treated cells.



The Normal Probability Plot conforms to a normal distribution of the variables

APPENDIX 5

Statistical analysis (ANOVA and Paired Sample t Test) for the dynamic equilibrium of the cortical ER

A Paired-Sample t Test was used for the cortical ER covered in the non-growing untreated cells over a period of 60 seconds. The total area covered by the cortical ER was measured using the ImagePro software and analyzed initially using the ANOVA: One way as only one factor, the area of cortical ER was considered. The results of the analyses are set out in the tables below.

Table A.5.1 ANOVA for area covered by cortical ER at α = 0.05

SUMMARY

Groups	Count	Sum	Mean	SD	Variance
Area of cortical ER in non-growing cells at 0 seconds	10	504.21	50.421	17.074	291.52
Area of cortical ER after 60 seconds	10	506.83	50.683	16.293	265.47

ANOVA α = 0.05

Source of variation	SS	DF	MS	F	P-value
Between groups	0.34	1	0.343	0	0.9724
Within groups	5012.87	18	278.493		
Total	5013.21	19			

The area covered by the cortical ER differs between the cells at a significant level of 0.1. It is necessary to perform the Tukey Test to determine where the difference lies.

TABLE A.5.2 Paired-Sample t Test for dynamic equilibrium of cortical ER at α =0.05

H_O **:** There is no difference between the area of the cortical ER over a time period

H_A **:** There is a difference between the area of the cortical ER over time period

	Area of cortical ER at 0 seconds	*Area of cortical ER at end of 60 seconds*
Mean	50.4196	50.68329649
Variance	291.5443412	265.4904071
Observations	10	10
Pearson Correlation	0.992957372	
Hypothesized Mean Difference	0	
df	9	
t Stat	-0.391863816	
P(T<=t) two-tail	0.704276023	
t Critical two-tail	2.262157158	

Conclusion: Since t< t Critical, H_A is rejected. The average area of cortical ER over a time period remains constant at a significance level of 0.05

APPENDIX 6

Statistical analysis (Paired Sample t Test) for difference in the cell size of non-growing and auxin treated cells

Table A.6.1 Difference in the cell size between non-growing and auxin treated cell

H_O: There is no difference in the length of the non-growing cells treated with auxin for 24hours

H_A : There is difference in the length of the non-growing cell treated with auxin for 24 hours

Cell Length

t-Test: Two-Sample Assuming Equal Variances

	Length of non-growing cell	*Length of same cell after auxin treatment*
Mean	7.3	12.047
Variance	1.139866667	1.32624556
Observations	10	10
Pooled Variance	1.233056111	
Hypothesized Mean Difference	0	
df	18	
t Stat	-9.55900775	
P(T<=t) two-tail	1.77966E-08	
t Critical two-tail	2.100922037	

Conclusion : Since t < -t Critical, H_O is rejected. There is a difference in the length of the cell treated with auxin for 24 hours

Table A.6.2 Difference in the cell size between non-growing and auxin treated cell

H_O: There is no difference in the area of the non-growing cells treated with auxin for 24hours

H_A: There is difference in the area of the non-growing cell treated with auxin For 24 hours

Cell Area

t-Test: Two-Sample Assuming Equal Variances

	Area of non-growing cell	*Area of same cell after auxin treatment*
Mean	21.717	36.753
Variance	15.99678	26.12358
Observations	10	10
Pooled Variance	21.06018	
Hypothesized Mean Difference	0	
df	18	
t Stat	-7.32633	
P(T<=t) two-tail	8.38E-07	
t Critical two-tail	2.100922	

Conclusion : Since t < -t Critical, H_O is rejected. There is a difference in the area of the cell treated with auxin for 24 hours